Establishment of a Novel *Pichia Pastoris* Host Production Platform

by
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B.S., Biomedical Engineering, University of Rochester, 2012

Submitted to the MIT Sloan School of Management and the Department of Mechanical Engineering in partial fulfillment of the requirements for the degree of

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ABSTRACT

The Cell Line Development group at Amgen is responsible for manufacturing and optimizing the cell lines utilized in production of Amgen's biologic drug portfolio. Traditionally, these cell lines are produced from mammalian host organisms, primarily Chinese Hamster Ovary (CHO) cells, due to their unique ability to secrete human-like glycosylated proteins. The CHO platform has undergone significant optimization throughout the industry over the past 30 years, however, productivity and efficiency improvements are now becoming harder to realize.

Alternative hosts offer a unique opportunity to drive significant cost of goods improvements throughout the biologic drug manufacturing process. Microbial hosts benefit from low genomic complexity, fast doubling times, and can grow to high cell densities in low-cost media. The yeast strain, *Pichia pastoris*, combines these advantages with the ability to secrete glycosylated products at equivalent product quality levels as CHO-based processes. The Alternative Host Consortium, an MIT-industry partnership, is focused on the advancement of *Pichia* and other alternative hosts to eventually drive broader commercial utilization and help curb the rising cost of biologic medicines.

This project aimed to quantify the strategic advantage of the *Pichia* host in Amgen's pipeline, and determine when, why and how such a product would be manufactured. The first segment of the work presented here includes various bioprocess development experiments performed to establish proof-of-concept protein production data in *Pichia*. The results show successful production of two relatively simple proteins at concentrations similar to existing published results. Additionally, chemically defined media and controlled fed-batch fermentation experiments were run to better mimic manufacturing scale operations. The second segment of the project focused on quantifying the strategic cost advantage of the *Pichia* platform compared with CHO. The business case analysis centered on potential raw material and plant time savings to determine the critical *Pichia* process features required to be cost competitive.

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I feel fortunate that I was able to work on an internship project where I felt meaningfully invested in the outcome of the work. The research being done by the Alternative Host Consortium is extremely exciting for potential future modes of biomanufacturing, and I'm happy to have contributed in a small way towards their mission. I want to sincerely thank the entire Amgen team for their time and guidance. Hedieh Barkhordarian was an immediate advocate for me, and I will continue to value her mentorship well beyond this project.

I also value the input of my thesis advisors, readers and the LGO support staff. Having Ketan Kumar and Ellie Azolaty as co-workers, classmates, roommates and friends undoubtedly made our 6 months in California all the more fun. I continue to be supported by a much cherished circle of family and friends, without whom I certainly wouldn't have been afforded any of the incredible opportunities that have come my way.

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GLOSSARY

AB	Antibiotic
BMGY	Buffered Glycerol-complex media
BMMY	Buffered Methanol-complex media
CCF	Cell culture fluid
CHO	Chinese hamster ovary
CLD	Cell line Development
COGM	Cost of goods manufactured
ELISA	Enzyme-linked immunosorbent assay
G-CSF	Granulocyte colony stimulating factor
HAS	Human serum abumin
HCP	Host cell protein
mAb	Monoclonal antibody
MCB	Master cell bank
OD	Optical density
OUR	Oxygen uptake rate
PD	Process Development
PTM	Post translational modification
R&D	Research & Development
RDM	Rich Defined Medium
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
WCB	Working cell bank
YPD	Yeast Extract-peptone-dextrose

1. BACKGROUND AND INTRODUCTION

1.1. INDUSTRY OVERVIEW

The biopharmaceutical industry is focused on harnessing the capabilities of living systems towards the development of life saving medications. Protein therapeutics, or biologics, are large molecular weight substances produced by a variety of living cells. Biologics can be used to treat any number of diseases where the underlying disease pathophysiology depends on known protein interactions. Patients with specific protein deficiencies or mutations often experience debilitating or degenerative disorders resulting from the breakdown of underlying metabolic pathways. By providing working copies of specific proteins therapeutically, patients can overcome these disease pathways despite their underlying conditions. The first biologic, recombinant human insulin, is a simple but incredibly powerful example of this type of treatment for the millions of insulin-deficient diabetes patients worldwide [1].

While some protein therapeutics are naturally produced by existing living systems, more often the host cells need to be manipulated to generate the protein of interest. Recombinant DNA technology allows scientists to insert specific DNA sequences into host cells, thus providing instructions for the production of their target protein. These cells are then grown to high concentrations in large bioreactors where they secrete vast amounts of protein that can be harvested and purified into doses of biologic therapeutics suitable for administration in humans. Such recombinant proteins represent a majority of commercially available biologics, a global market expected to value to nearly 400 Billion USD by 2025 [2].

Amgen is one of the world's leading biotechnology companies, offering a portfolio of biologic medicines for the treatment of cardiovascular disease, bone disease, inflammation,

oncology and nephrology. Headquartered in Thousand Oaks, CA, Amgen employs nearly 20,000 staff worldwide to bring its life-saving medications to a global community of patients. The transformation of a given target protein into a viable human therapeutic occurs largely through the interaction of Amgen's Research and Development (R&D) and Process Development (PD) functions. When a target genetic sequence is identified by R&D as a potential product candidate, the responsibility falls to the Cell Line Development group within PD to engineer a manufacturing host cell line to create a robust and high producing master cell bank . The focus of this research centers on the engineering of appropriate host cells that can reliably, efficiently and cost-effectively produce Amgen's pipeline of biologic therapies to meet an ever-growing global demand.

1.2. PURPOSE OF PROJECT / PROBLEM STATEMENT

The vast majority of recombinant proteins at Amgen, and throughout the biopharmaceutical industry, are produced in one type of host cell; Chinese Hamster Ovary cells (CHO) . Due to their mammalian origin, CHO cells have the unique ability to secrete human-like glycosylated proteins. The CHO platform has undergone significant optimization throughout the industry over the past 30 years, however, productivity and efficiency improvements are now becoming harder to realize [2], [3].

Meanwhile, global demand for biopharmaceuticals continues to rise. The promise of therapeutic and prophylactic treatments for infectious or gastrointestinal diseases could generate enormous new patient populations [3]. In addition to increased demand, the current cost of biologics is prohibitive for treatment of patients in emerging markets. Current CHO

biomanufacturing therefore faces capacity and cost pressures with a host cell system that has already been pushed to the extreme limits of its performance capability.

Alternative hosts offer a unique opportunity to drive significant improvements in cost of goods throughout the biologic drug manufacturing process. Microbial hosts benefit from low genomic complexity, fast doubling times, and can grow to high cell densities in low-cost media [4]. The yeast strain, *Pichia pastoris*, combines these advantages with the ability to secrete glycosylated products at equivalent product quality levels as CHO-based processes. The Alternative Host Consortium, an MIT-industry partnership, is focused on the advancement of *Pichia* and other alternative hosts to eventually drive broader commercial utilization and help curb the rising cost of biologic medicines.

Through the partnership with the Alternative Host Consortium, this project aimed to better assess the role that a *Pichia* host platform might play in Amgen's drug development pipeline. The problem statement was to assess the current performance capability of the *Pichia* host compared with CHO through protein expression experiments. By generating proof-of-concept data for the *Pichia* host system, we would bring yeast cultivation techniques in-house at Amgen to establish capabilities for future testing alongside CHO. Additionally, this project aimed to better quantify the potential for cost-reduction using *Pichia*, and specifically identify where savings could be realized in the product development, manufacturing and commercialization process.

1.3. PROJECT GOALS

This project aimed to quantify the strategic advantage of the *Pichia* host in Amgen's pipeline, and determine when, why and how such a product would be manufactured. The capabilities of *Pichia* can be examined through prior research results or existing *Pichia*-based

commercial manufacturing processes. However, those resources offer little practical understanding of the implications of what a *Pichia* system would entail if implemented at Amgen. This project represented a first step towards building the practical capabilities for *Pichia* production in house in Thousand Oaks.

Establishing Pichia cultivation capabilities

The first goal upon arrival at Thousand Oaks, was to work with the Cell Line Development (CLD) team to establish the ability to cultivate *Pichia* cultures in-house. While some legacy products utilized *E. coli* expression systems, the majority of ongoing CLD activities were executed in CHO host systems. *Pichia* specific materials, lab space, segregation controls and analytical testing capabilities needed to be established prior to beginning any wet-lab testing.

Provide initial proof-of-concept protein expression results

Once the initial setup was complete, our goal was to provide a robust set of proof-of-concept data to inform *Pichia's* current performance level. Utilizing materials and procedural guidelines provided by the Alternative Host Consortium, we would execute our own protein expression experiments to validate and build upon prior published results. The vector and base strains provided by the consortium for these experiments were composed of basic components commonly used for *Pichia* protein expression. Additional optimization of these components is ongoing and aims to produce highly engineered strains that maximize expression.

Inform high impact areas for implementation

Alongside wet-lab testing, another critical goal was to better investigate the business implications of a *Pichia* host system. The driver was to establish a Framework to compare estimated *Pichia* costs with existing CHO costs. This analysis would work to inform where in the pipeline *Pichia* could provide the highest cost reduction impact and specify where the savings would be realized throughout the product commercialization lifecycle.

1.5. PROJECT APPROACH

The structure of the project materialized into two distinct objectives: (1) *Pichia* process development; (2) quantifying the strategic cost advantage of *Pichia*, if any. The information and expertise required to support these two objectives came from different groups of people within Amgen. My immediate management within Cell Line Development fully supported the process development and wet-lab testing segment of the work. Leveraging expertise of the Amgen scientists with the best practices provided by prior publications and the Alternative Host consortium, we were able to quickly establish *Pichia* cell lines to begin experimental testing. The business case analysis, however, required expertise from resources outside of the PD group. I established a working team of advisors from various functions including operations strategy, process planning, engineering and supply chain management. Using their guidance, I was able to build an appropriate business case framework and access the necessary cost models to perform the analysis.

2. LITERATURE REVIEW

2.1. RECOMBINANT PROTEIN PRODUCTION IN *PICHIA* VS. CHO

The cell line development process for *Pichia* follows a largely standardized approach for microbial recombinant protein expression. The figure below highlights the major steps of the process, which will be discussed in detail in the Research Methodology section later on. A critical

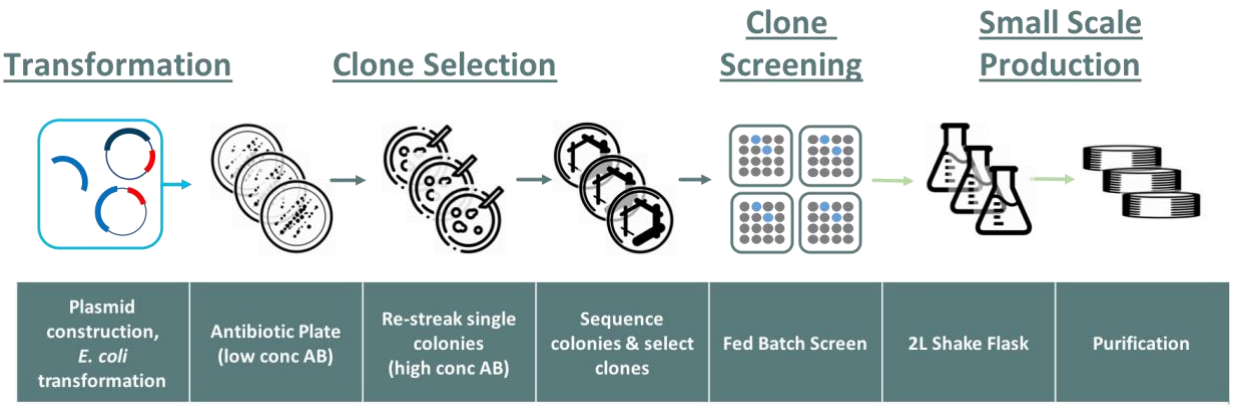

FIGURE 1. PICHIA CELL LINE DEVELOPMENT PROCESS SUMMARY (AB: ANTIBIOTIC)

difference between *Pichia* and CHO cells during this process is their response to transformation/transfection. During these steps, the cell wall is stressed through methods like electroporation to allow the gene-containing vector to enter the cell. While both types of cells require recovery from this process, CHO cells take weeks to return to pre-transfection viability levels, whereas *Pichia* cells can recover from transformation in a matter of hours [5].

Additional factors contribute to the speed of generating a *Pichia* cell line compared with CHO. The most critical, potentially, is the doubling time. Where a *Pichia* cell will double in ~2 hours, a CHO cell will require 19-25 hours [3]. This doubling speed accounts for faster growth at every stage of the fermentation process. Per estimates outlined in the figure below, *Pichia* is capable of shortening timelines vs CHO by a factor of months in the end-to-end cell line

development process. One critical advantage contributing to the faster cycle times is that the *Pichia* genome is ~200 times smaller than CHO cells [2]. Smaller genome size allows for faster and cheaper sequencing processes that contribute to shorter timelines for cell line genetic characterization [4].

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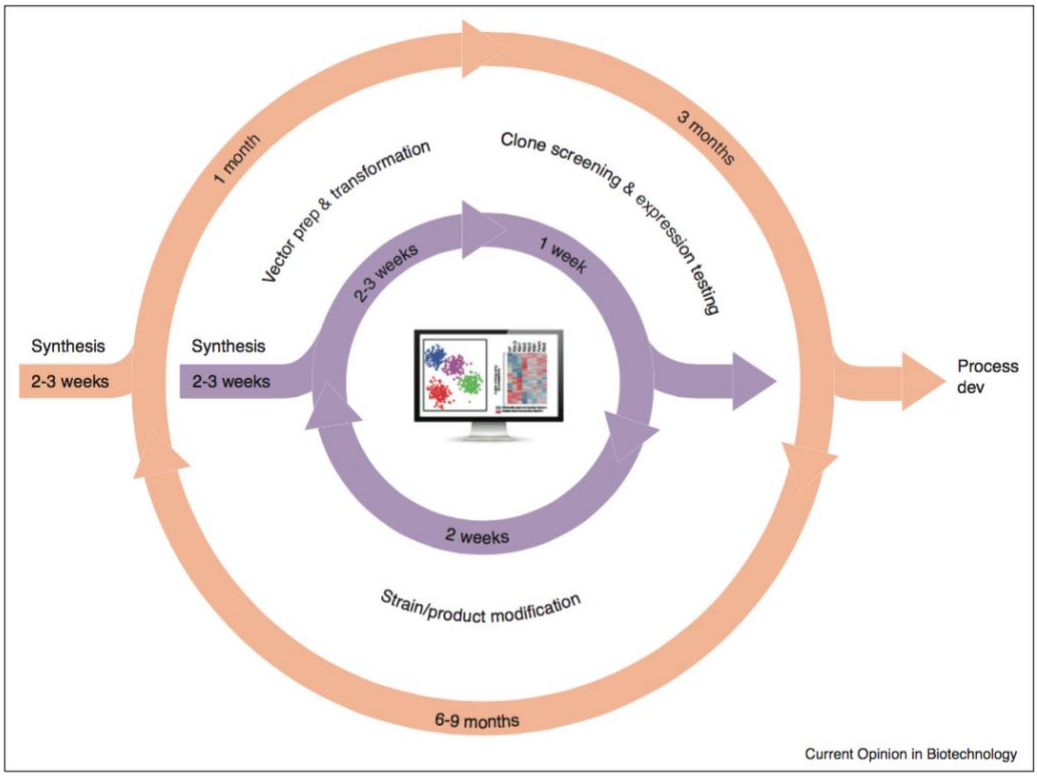

Host strain development timelines. Strain iteration for is shown for yeast (purple) and for CHO (orange).

Figure 2. TIMELINE ESTIMATES TAKEN FROM FIGURE 2 IN LOVE ET. AL, 2018

[2]

Despite its speed, *Pichia* also has inherent limitations that have prevented its broader use in commercial biologics manufacturing thus far. A main concern is the inability to naturally achieve human-like post-translational modifications (PTMs), such as glycosylation of secreted proteins [2], [3]. This limitation has been challenged, however, by a number of examples of innovative strain engineering. Past scientists, as well as current Alternative Host Consortium

researchers, are working to directly incorporate the enzymatic machinery required to produce human-like glycosylation alongside therapeutic genes of interest [2], [6].

2.2. *PICHIA* HISTORICAL PERFORMANCE

Aside from glycosylation profiles, another limitation of *Pichia* compared to the production of MAbs in CHO is the ability to secrete these biologics at viable protein concentrations for commercial processing. The figure below outlines relative productivity of a variety of proteins in

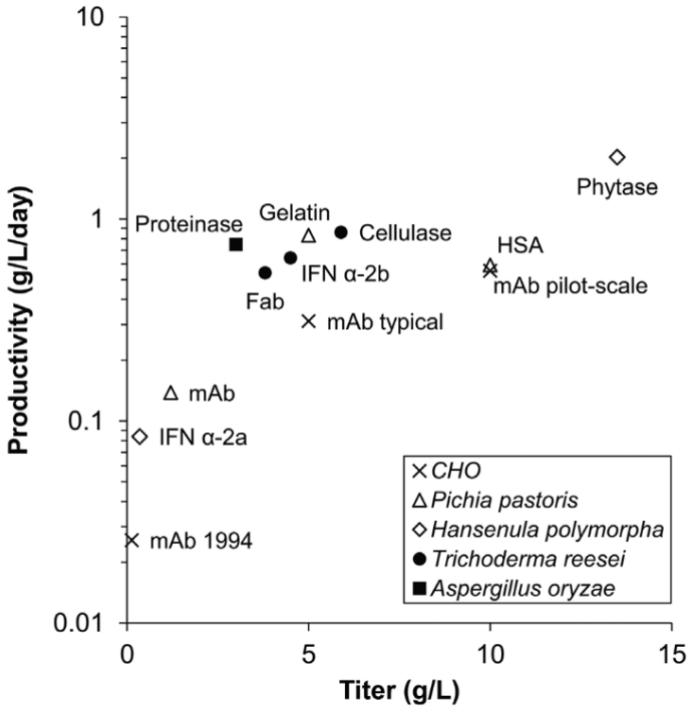

FIGURE 3. COMPARISON OF FERMENTATION PERFORMANCE BY HOST FOR VARIOUS PROTEINS PER MATTHEWS ET. AL, 2017 [6].

various host systems [4]. While Pichia shows high productivity for less complex molecules, mAB productivity is significantly lower than what is achieved from modern CHO processes. Other sources cite CHO perfusion cultures acheiving 2 g/L/day in monoclonal antibody (mAb) production, in comparison from the *Pichia* estimate of ~0.15 g/L/day [3].

However, despite the glycosylation and productivity challenges, there have also been notable successes reported in the literature. In 2008, a Merck subsidiary called Glycofi achieved the results outlined in the figure below for production of a glycosylated mAb in *Pichia* [7]. Through an intensive strain engineering effort, Glycofi, was able to produce the cited mAb product with uniform glycan structure. The functionality of the glycosylation was further tested for therapeutic efficacy which matched the associated marketed product. Additionally, through process

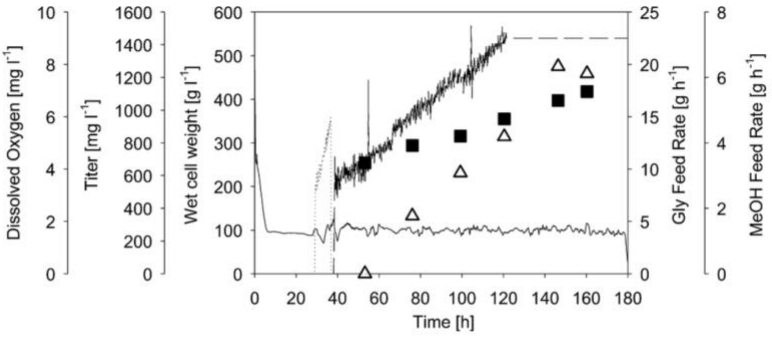

Fig. 1. Cultivation of YGLY4140 at 3-l scale. Biomass concentration as measured by wet cell weight in $g l^{-1}$ as a function of the cultivation time is indicated by the symbol: (■). Antibody titer after capture by rProteinA relative to the broth volume in $mg l^{-1}$ as a function of cultivation time is indicated by the symbol (△). The solid line indicates the dissolved oxygen concentration in $mg l^{-1}$. The dotted line (···) indicates the 50% glycerol feed rate in $g h^{-1}$ while the dashed line (- - -) indicates the methanol feed rate in $g h^{-1}$.

FIGURE 4. GLYCOFI CULTIVATION RESULTS FOR GLYCOSYLATED MAB PRODUCT IN PICHIA, FIGURE 1 FROM POTGEITER ET. AL [7].

optimization techniques that included optimized oxygen uptake rate (OUR) control and methanol feeding strategies, they were able to achieve ~0.2 g/L/day production at the 3L fermenter scale.

These results drive home the potential promise of the *Pichia* host given significant investment in strain engineering and process optimization. The Alternative Host Consortium is founded on the idea of pulling industry experts together with MIT researchers to drive a collected effort towards maturation of *Pichia* and other potential alternative hosts. The early results of that partnership are further tested in the research presented here.

3. RESEARCH METHODOLOGY

This section describes the methods and rationale used during various wet-lab *Pichia* cultivation experiments. It is divided into 3 sections: (1) Methodology for *Pichia* cultivation from cloning through small-scale shake flask batch processes; (2) Rationale for defined media testing; (3) Rationale for fed-batch fermentation testing. These experiments were executed using two different molecules; G-CSF and Protein1. There are several prior published results for G-CSF grown in *Pichia*, which allowed for head-to-head benchmarking of our data. Protein1 was chosen due to prior published results in microbial hosts. Its relative simplicity and low molecular weight gave confidence that we could produce measurable protein concentrations in *Pichia.* The intent of producing these two molecules was to show proof-of-concept data for the *Pichia* host, and to establish small-scale microbial process capabilities for the Cell Line Development group.

3.1. METHODOLOGY FOR PICHIA CULTIVATION

The methods used for the small-scale process testing were based on standard operating procedures provided by the Alternative Host Consortium. These procedures outlined best practices for cloning, high throughput screening and shake flask batch process testing.

3.1.1. CLONING AND TRANSFORMATION

All experiments were conducted using wild-type *Pichia* strain, Komagataella phaffii (NRRL Y-11430). A frozen stock of wild type cells was used to create master cell banks of each protein expressing strain; G-CSF and Protein1. Plasmid vectors provided by the Alternative Host Consortium were used to incorporate the gene(s) of interest into the host cells. The genetic

sequence for each protein was codon-optimized for expression in *Pichia* using Thermofisher's web-based "Gene Synthesis" tool. The codon-optimized sequence for G-CSF was taken from prior published research and is shown in Table 1.

TABLE 1. G-CSF GENETIC SEQUENCE[8]

G-CSF	Codon Optimized DNA sequence
	ATGACTCCTTTGGGTCCAGCTTCTTCCTTGCCTCAATCCT TCTTGTTGAAGTGTTTGGAGCAGGTTAGAAAGATCCAGG GTGATGGTGCTGCTTTGCAAGAGAAGTTGTGTGCTACTTA CAAGTTGTGTCACCCAGAAGAGTTGGTTTTGTTGGGTCAC TCCTTGGGTATTCCTTGGGCTCCATTGTCCTCTTGTCCAT CCCAAGCTTTGCAATTGGCTGGTTGTTTGTCCCAATTGC ACTCCGGTTTGTTCTTGTACCAGGGTTTGTTGCAAGCTTT GGAGGGTATTTCTCCAGAGTTGGGTCCAACTTTGGACACA TTGCAGTTGGACGTTGCTGACTTCGCTACTACTATCTGGCAA CAGATGGAAGAATTGGGTATGGCTCCAGCTTTGCAGCCAACTC AAGGTGCTATGCCAGCTTTTGCTTCTGCTTTCCAGAGAA GAGCTGGTGGTGTTTTGGTTGCTTCTCACTTGCAGTCTTTC TTGGAGGTTTCCTACAGAGTTTTGAGACACTTGGCTCAACCA

Two vectors containing different antibiotic resistance markers were used to generate the vector constructs for each protein strain, see Table 2.

TABLE 2. ANTIBIOTIC RESISTANCE MARKERS

Protein Strain	**Antibiotic Resistance marker**
G-CSF	Geneticin
Protein1	Zeocin

The vectors each also contained an α-factor secretion signal under control of the methanol-induced AOX1 promotor. This promotor is commonly used in *Pichia* cultivation and works to drive production of target proteins in the presence of methanol.

The appropriate antibiotic vector backbone(s) and codon-optimized gene insert(s) were ligated by Gibson Assembly. Each integrated vector was transformed into *E. coli* cells. These cells

were then incubated on agar plates with media containing a low concentration of the appropriate antibiotic. Only cells that successfully incorporated the resistance marker, as well as our gene of interest, survived this incubation. Individual clones were then isolated by harvesting single surviving colonies from the antibiotic plates and resuspending them in culture. In turn, the new gene-containing vector was isolated from the *E. coli* cells and underwent Sanger sequencing to confirm the proper integration of the target gene.

After confirming each vector contained the functional genetic sequence for each protein, we proceeded with transformation of *Pichia* wild type cells. Prior to this process, a stock of electrocompetent *Pichia* cells was generated from the frozen wild type stock. We then used electroporation to introduce the vector into the cells. This process involves applying a brief electrical field to momentarily increase cell permeability and allow the vector to enter the interior of the cell. Cells were then briefly incubated in a recovery mixture before again being spread on agar plates with media containing the appropriate antibiotic.

After a 2-3 day incubation, several *Pichia* colonies had formed. To establish a cell stock containing only a single clone, individual colonies were selected and re-streaked onto another set of agar plates. To select for cells with the highest uptake of our target sequence, we increased the concentration of antibiotic present in the agar media 4-fold. After another 2-3 day incubation period, colonies present on the high concentration antibiotic plates were sampled to perform Sanger sequencing. Once sequencing results confirmed the correct target genetic sequence was successfully incorporated, the colony material was mixed with liquid media in a cryogenic vial. These vials were then frozen and stored as the master cell banks (MCB) for each protein strain. Multiple clones underwent this process, so duplicate MCB vials were generated for 30 different clones of each protein.

3.1.2. CLONE SCREENING AND SELECTION

High throughput clone screening is used as an initial test of protein production performance of newly made strains. We performed this process for 8 different clones for each protein. Based on the results, we selected 2 high performing clones to carry forward into small scale batch testing.

Each well of a 24-well plate was filled with basic Yeast Extract-Peptone-Dextrose (YPD) media. For a complete list of media components and concentrations, please reference the Appendix. Wells were then inoculated with a small sample from the MCB of each clone being tested. The clones were tested in triplicate to ensure backup samples in the case of contamination. Maps of the 24-well plates for each protein are included here. The number indicates the clone number assigned during MCB creation. One well was used as a positive control and was filled with a sample of a confirmed protein-containing strain. This strain was provided by the Alternative Host Consortium and contained Human Serum Albumin (HSA).

TABLE 3. G-CSF CLONE SCREENING PLATE MAP

17	17	17	21	21	21
18	18	18	22	22	22
1	1	1	23	23	23
20	20	20	27	27	Control

TABLE 4. PROTEIN1 CLONE SCREENING PLATE MAP

1	1	1	14	14	14
4	4	4	21	21	21
9	9	9	27	27	27
12	12	12	28	28	Control

The prepared plates were then incubated overnight on an orbital shaker. The following day, the cell density in each well was determined by an optical density reading. A spectrophotometer (Eppendorf Biospectrometer) was used to record an OD_{600} measurement for each well (OD_{plate1}) .

A separate 24-well plate was prepared with Buffered Glycerol-complex media (BMGY) media (see Appendix for media component breakdown). This media provides glycerol as the carbon source to drive cell growth. To meet a target initial optical density (OD_{target}) in the new plate, the appropriate inoculation volume (V_{inoc}) was determined based on the following formula:

$$V_{inoc} = \frac{V_{working} OD_{target}}{OD_{plate1}}$$

Once inoculated, the plates were incubated for 24 hours on an orbital shaker.

After one day of growth in glycerol media, another optical density measurement was taken. The BMGY media was then exchanged with Buffered Methanol-complex media (BMMY), see Appendix for media component breakdown. Switching the carbon source from glycerol to methanol shifted the cells from a replication/growth state towards a protein production state via the AOX1 promotor. Plates were incubated on an orbital shaker for an additional 24 hours, after which a final OD_{600} reading was recorded.

To complete the run, the protein produced in each well needed to be separated from the spent cells and analyzed for protein concentration levels. The plates were centrifuged to separate the solid cell material from the protein containing cell culture fluid (CCF). The CCF was then aliquoted for further sampling. To measure the protein concentration produced by each clone, we utilized SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) analysis. This

method separates proteins based on their molecular weight and provides a means for estimating sample protein concentration with respect to a standard. Samples from the CCF of each clone were

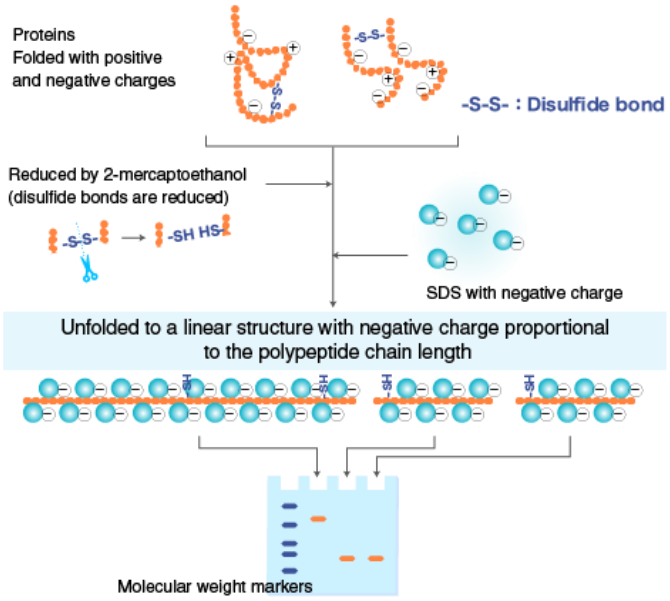

FIGURE 5. SDS-PAGE GEL OVERVIEW[9]

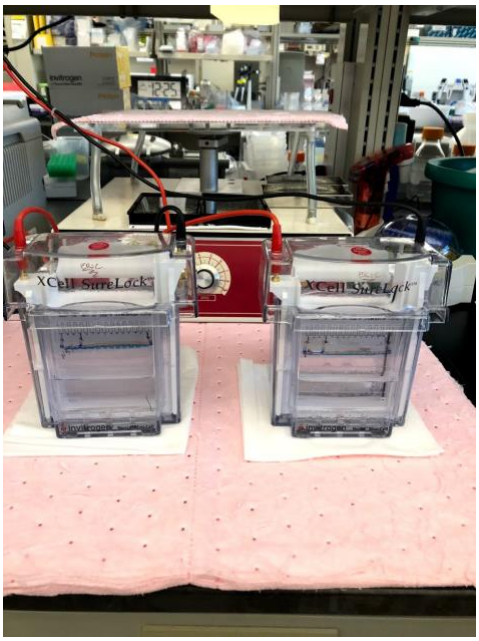

IMAGE 1. SDS-PAGE GEL LABORATORY SETUP

loaded into separate pens of a gel matrix, see Figure 5. An electric field is then applied across the gel, and proteins migrate from cathode to anode at speeds relative to their molecular weight. A DNA "ladder" is also run along the gel to provide a reference standard for known molecular weights. Lastly, reference standard material for each protein (G-CSF, Protein1) was run alongside the CCF samples. These standards were run at multiple concentrations, with higher concentrations producing increasingly dark bands along the gel. The reference bands allowed for estimation of relative protein concentration produced throughout the run by each of the clones. The experimental setup for the SDS-PAGE gel analysis is shown in Image 1. The two clones that produced the darkest bands, and therefore highest protein concentrations (mg/L), were selected to continue with further small scale testing.

3.1.3. SMALL SCALE CULTIVATIONS

Working cell banks (WCB's) were created from the MCB stock for each high performing clone. WCB aliquots were controlled such that one WCB cryovial could directly inoculate a 1L shake flask at the appropriate initial cell concentration. Shake flask cultivations were performed in 1L sterile plastic baffled flasks (Fisherbrand #BBV1000). One flask was assigned per protein and clone (i.e. G-CSF, clone 21). BMGY media was warmed to room temperature and added to each flask. The WCB cryovial was removed from storage and allowed to thaw on ice. Once evenly thawed, each WCB was added to its respective flask and an initial OD_{600} reading was taken. The cultures were then incubated for 24 hours in a shaking incubator.

After 24 hours in BMGY growth media, another OD_{600} reading was taken to assess cell density. Over the first day of growth, the cells consumed the initial supply of glycerol and the media was exchanged to supply an additional carbon source. To remove the spent BMGY media, the culture was centrifuged in a swinging bucket centrifuge. The supernatant was decanted and disposed. A fresh supply of BMMY production media was then added to the remaining cell pellet. The cells were gently resuspended into solution and the culture was transferred to a fresh shake flask. Cultures were then run in a shaking incubator for another 24 hours in the methanol-containing production media.

Another OD_{600} reading was taken after 24 hours in BMMY. At this point, the run could either be terminated or an additional supply of methanol could be added to try to drive further protein production. If a methanol spike was added, the culture would run for an additional 24 hours under the same shaking incubator conditions. Once the run was completed (with or without methanol spiking), the culture would be harvested and supernatant samples collected for further testing. As previously described, the culture would be centrifuged to separate the solid cell material

from the protein-containing supernatant. The supernatant was then collected and stored for SDS-PAGE analysis.

3.2. RATIONALE FOR DEFINED MEDIA TESTING

Media formulations are critical to the growth and productivity of cells in any biopharmaceutical process, microbial or mammalian. Media raw material components can be classified as either “defined” or “complex” depending on their underlying chemistry. Defined materials are composed entirely of biochemicals with known compositions. Complex materials come from biological origins and can contain a variety of animal or plant-based compounds such as serum, peptone, or yeast extract [10]. Due to their biological make-up, complex materials are inherently variable in their composition. When used in media formulations, this variability can lead to batch-to-batch performance variations which can be costly to address in quality and regulatory documentation. Defined media formulations have therefore been heavily developed in CHO manufacturing to improve process stability and reduce regulatory complexity [10].

There has been less effort to develop defined media formulations for *Pichia* processes. The standard complex media used for *Pichia* cultivation is buffered complex glycerol medium (BMGY). BMGY components and relative concentrations are listed in Appendix A.1. The formulation contains two complex raw materials; peptone and yeast extract. Protein hydrolysis and cell autolysis are used to form these components. These ingredients carry the same variability and regulatory risks as complex CHO media components described above.

Additional consideration should also be given to the potential risk of viral contamination from complex raw materials in *Pichia* processes. CHO processes contain viral inactivation and filtration steps to ensure any viral adventitious agents have been sufficiently eliminated from the final product. These viral contaminants could be harbored by the CHO cells during cell culture, or

could be introduced in raw materials. However, *Pichia* fermentation differs from CHO cell culture in that it does not support the propagation of viruses, and therefore does not require the same viral clearance process steps [11]. The elimination of these steps reduces both operational durations and production costs for *Pichia* processes in comparison with CHO. However, these savings can only be realized if the risk of viral contamination from raw materials has also been eliminated. Complex raw materials of biological origin should therefore not be included in *Pichia* media if viral clearance steps will not be included in the operational process [11].

Researchers at the MIT Koch Institute developed a defined media formulation for *Pichia* fermentation. Using systematic nutrient evaluation and transcriptomics, they developed the "Rich Defined Media" (RDM) formulation outlined in Appendix A.1. In their research, this formulation was able to match and even outperform complex media (BMGY) in cell growth and protein production for certain molecules [10]. Here, we attempted to repeat their results using the shake flask cultivation methods described above for production of G-CSF in *Pichia*.

The G-CSF working cell banks described in section 3.1.3 were utilized for media comparison testing. The aim of the experiment was to compare growth and G-CSF protein production performance for identical clones cultivated in complex media (BMGY) versus Rich Defined Media (RDM). G-CSF clone 20 was used for this test. First, RDM was prepared according to the formulation published in Matthews et. al and outlined in Appendix A.1. There were two versions of the media; one for outgrowth, one for production. The outgrowth media contained glycerol as the carbon source similar to BMGY. The production media contained methanol as the carbon source similar to BMMY.

Two day shake flask cultivations were performed according to section 3.1.3. For the RDM cultivation, outgrowth media was used in place of BMGY. On the final day of cultivation,

Production RDM media was used in place of BMMY. All measurements including OD_{600} and protein concentration by SDS-PAGE were performed according to the previous section.

3.3. RATIONALE FOR SMALL SCALE FERMENTER TESTING

Batch shake flask cultivations are fairly limited in the level of process control compared with larger scale fermenter systems. In a batch shake flask process, the only process parameters that are actively controlled are the temperature and agitation settings of the incubator, and media additions can only be made in manual bolus additions. In a standard bioprocess fermenter, however, additional process controls are available including dissolved oxygen, pH and controlled peristaltic feed pumps. The ability to better monitor the state of the process allows for tighter control and manipulation of the cell environment, leading to enhanced process performance. Our goal was to utilize a small scale fermenter system, the Eppendorf DASBox, to provide initial proof of concept data for the potential performance improvement in a controlled fermenter vs. a shake flask.

FIGURE 6. EPPENDORF DASBOX FERMENTATION SYSTEM [5].

The Eppendorf DASBox system, shown above, is a fermenter system designed to run anywhere from 4-24 vessels in parallel. We utilized vessels with a working volume of 250mL. The system provides precise control and feedback for all critical process parameters including the following; overlay/sparge gassing, four media addition feed lines, temperature, pH, dissolved oxygen, level and agitation [12].

One critical difference between the DASBox system and prior shake flask testing is the ability to run a fed-batch process vs. a batch process. Fed-batch fermentation is an adapted form of the batch processes described earlier. The key difference is that after an initial batch period, additional media/nutrients are added to the system for the remaining duration of the run. By adding fresh nutrients throughout the run, additional biomass is accumulated to support higher protein production [13].

To design the process control strategy for our fermenter run we referenced prior research by scientists at Merck who showed strong protein production results in *Pichia*. An automated control recipe was developed on the DASBox control system according to the following specifications [7]:

Temperature	24 ± 0.5 °C
pH	6.5 ± 0.1
DO	1.7 ± 0.1 mg/L
Agitation	300 rpm
Glycerol Feed	50% glycerol containing 12.5 ml/L PTM salts Feed initiated 30h post inoculation and continuing for 8h Exponential feed starting at 5.3 g/L/h; increasing by 0.08g/L/h
Methanol Feed	Feed initiated 30 min after completion of Glycerol feed Exponential feed starting at 2g/L/h; increasing by 0.01g/L/h
Harvest	Run terminated at 144h Cells harvested in centrifuge at 2400g for 15min

TABLE 5. EPPENDORF DASBOX FERMENTATION CONTROL STRATEGY

Prior to the fermenter process, a 2-day shake flask run was executed to provide inoculation material. Two WCB stocks were utilized for this experiment; G-CSF-clone20 and Protein1-clone1. The WCB supplies were grown in complex media over a 2-day batch shake flask process as described in section 3.1.3.

In preparation, the 250mL fermenters and support materials (media addition bottles, probes) were sterilized and autoclaved as needed. All probes, pumps and sensors were calibrated prior to use. BMGY media was batched at a 200mL working volume and adjusted to a process temperature of 24°C. The vessels were then inoculated from the shake flask solution at a 10% v/v inoculum to BMGY ratio. At the time of inoculation, the timer controlling the onset of the various media feeds was initiated. The system automatically controlled these feeds and all other process parameters listed in the table above. Every 24 hours, the vessels were manually sampled to perform offline analytical readings including OD_{600}, pH, and wet cell weight. The online vessel pH value was adjusted to the offline reading as needed. Samples were retained at various time points for protein concentration analysis. The recipe proceeded for 144 hours until termination. Cell broth was harvested and stored for further testing. Interim and final samples were analyzed for protein concentration using SDS-PAGE analysis.

4. PROCESS DATA & ANALYSIS

4.1. CLONING & TRANSFORMATION

The vectors containing the gene of interest for both G-CSF and Protein1 were generated by Amgen cell line development scientists. A stock of wild type *Pichia* strain Komagataella phaffii (NRRL Y-11430) provided by the Alternative Host Consortium lab was prepared for electroporation. Following electroporation, the cells were immediately introduced into a recovery solution. The solution was then spread on low antibiotic concentration agar plates. After 2-3 days of incubation, the plates showed growth of colonies, an example of which is pictured below.

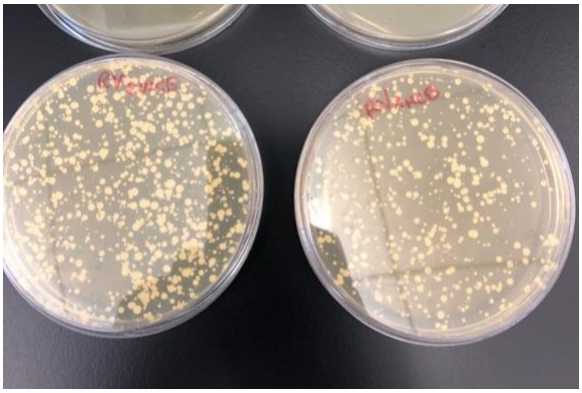

IMAGE 2. PROTEIN-1 COLONIES ON ZEOCIN CONTAINING AGAR PLATES.

Individual colonies that were large and fully isolated on the plates were selected for further processing. 30 individual colonies for each protein were harvested using inoculation loops and spread on high antibiotic concentration agar plates. Each plate was assigned a unique clone number. After 2-3 days of incubation in the presence of high concentration antibiotic, the plates showed colony formation, pictured below. The majority of clonal plates showed growth and duplicate MCB vials for each clone were generated from the material. Plates that showed indistinct colony formation were abandoned (<10% for each protein pool).

IMAGE 3. HIGH CONCENTRATION ANTIBIOTIC COLONY FORMATION - PROTEIN-1

High throughput screening to select individual clones was performed with a pool of eight clones for each protein. The clones were selected at random from the MCB pool. 24 deep-well plates, pictured below, were inoculated with each clone in triplicate. The plates were incubated on orbital shakers as described in section 3.1.2. OD_{600} readings were taken for each clone at 24

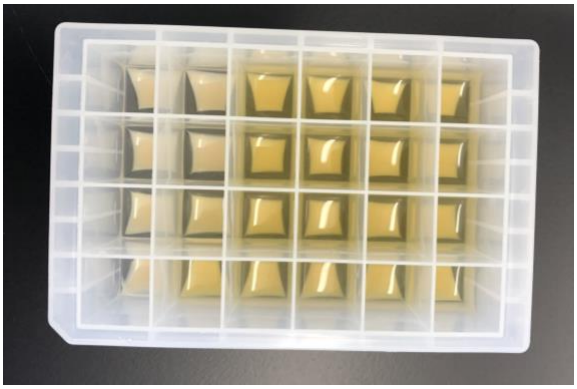

IMAGE 4. G-CSF 24 DEEP-WELL PLATE FOR CLONE SELECTION

hour increments. The growth trends for each molecule are shown below. The average OD level after 24 hours of growth was ~21.17, slightly higher than previously published microtiter plate cultures that reached OD levels of ~19 after 24 hours in BMGY[10]. Protein1 showed faster growth during the first 24 hours, whereas G-CSF grew more during the methanol induction period. The growth profiles each show strong consistency across clones with final OD standard deviation below 1.05. This consistency can help simplify the cell line development process in comparison

with CHO, where the level of clonal variability is routinely much higher and requires extensive forward processing of multiple clones to identify top performers [14].

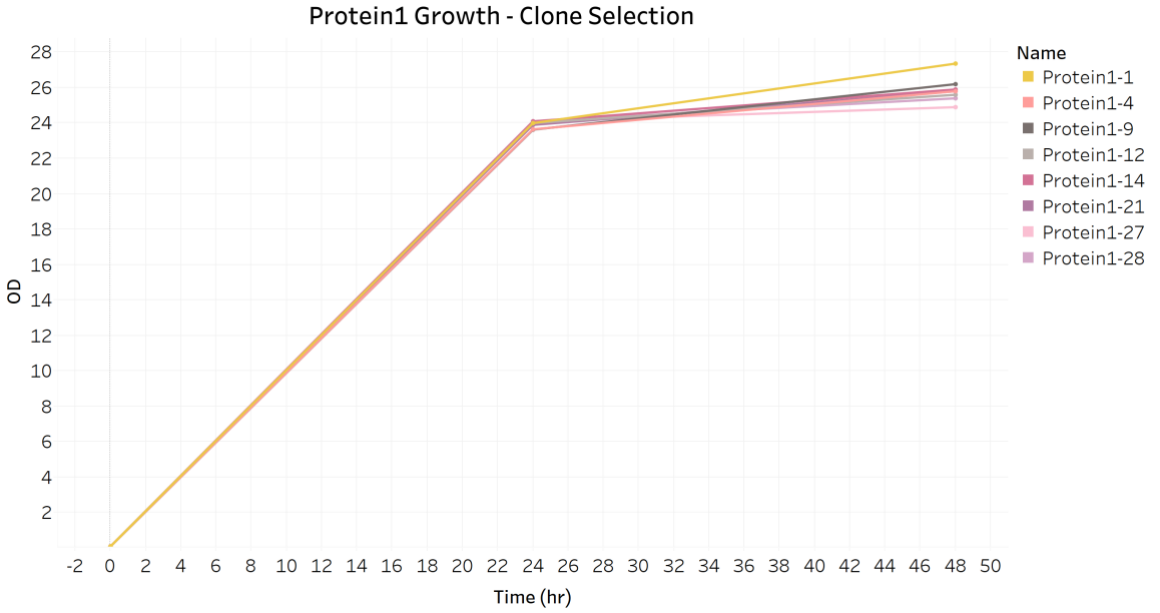

FIGURE 8. PROTEIN-1 CLONE SELECTION OD READINGS

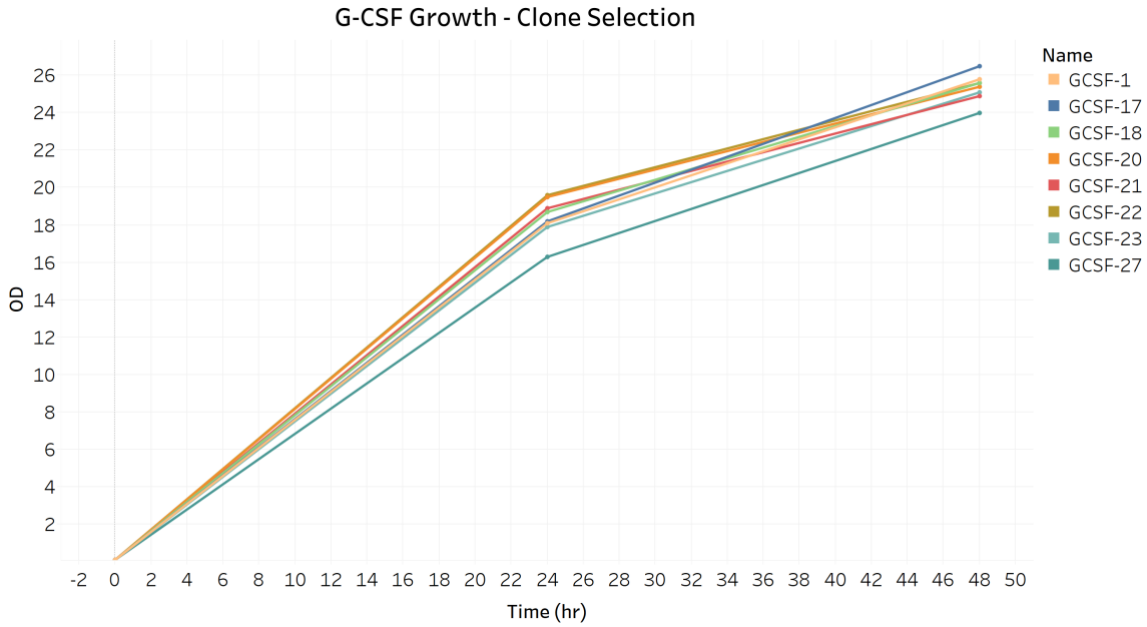

FIGURE 7. G-CSF CLONE SELECTION OD READINGS

At completion of the test, the protein-containing cell broth was harvested and underwent SDS-PAGE analysis to determine the amount of protein produced by each clone (measured as titer, mg/L). The following are images of the completed gels. The kilodalton scale on the left-hand side is a reference for the molecular weight of each species. A known reference standard for each protein was included on the gel. Two concentrations were included to act as a standard for estimation of the relative performance of each clone. The higher concentrations produce a darker band as more material aggregates at that level along the gel. A positive control containing human serum albumin was also included in the gel at two concentrations. This material was provided by the Alternative Host Consortium. Finally, the samples collected from each clone were run in individual lanes on the gel.

The G-CSF gel is shown below. The 8 clones show slight variation in their relative

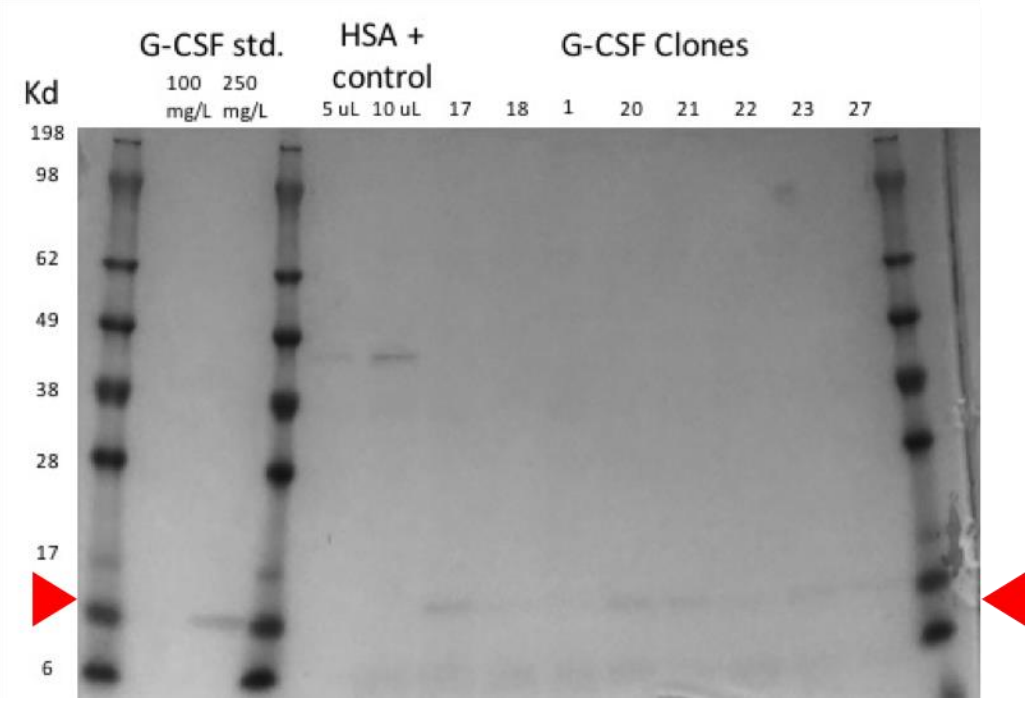

FIGURE 9. G-CSF CLONE SELECTION SDS-PAGE GEL

darkness, and therefore titer. The following three clones were identified as the strongest producers and were used going forward for shake flask testing:

GCSF clone	Estimated titer (mg/L)
20	200
21	150
23	150

TABLE 6. G-CSF TOP CLONE TITER ESTIMATES

The Protein1 gel is shown below and similarly shows slight variation in titer. There was an error during gel preparation on the 250 mg/L standard causing the material to be improperly loaded in

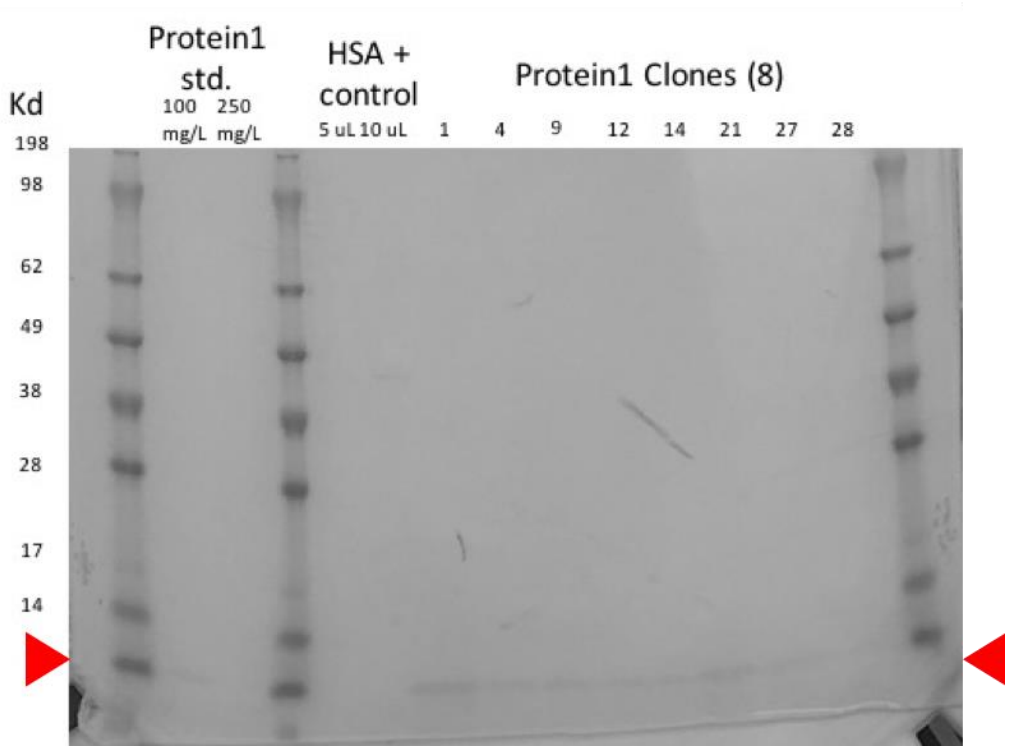

FIGURE 10. PROTEIN-1 CLONE SELECTION SDS-PAGE GEL

the well. The bar is therefore not visible for that concentration. The following 3 clones were selected for ongoing testing:

Protein-1 clone	Estimated Titer (mg/L)
1	200
9	150
21	200

TABLE 7. PROTEIN-1 TOP CLONE TITER ESTIMATES

Protein-1 achieved higher and relatively less variable titer results. This difference is potentially due to the smaller molecular weight and ease of expression compared to G-CSF, making the protein synthesis process easier to execute. It should be noted that SDS-PAGE titer analysis is a subjective analytical test. More formal analytical methods for titer assessment such as ELISA (enzyme linked immunosorbent assay) or mass spectrometry are used in clinical and commercial applications. These methods were not readily available for the molecules in this experiment. In future tests of potential drug candidates, these assays will likely be developed simultaneously and can be used for more accurate titer measurements.

4.2. SHAKE FLASK CULTIVATIONS

A working cell bank was created for each of the top 3 clones. Two clones per protein were then used for 1L shake flask testing. The shake flasks, pictured below, were baffled at the base. The baffles provide turbulent flow to the culture, creating bubbles that supply additional oxygen to the *Pichia* cells. Typical CHO cultures require less oxygen and a gentler agitation

IMAGE 5. 1L SHAKE FLASK BATCH CULTIVATIONS

mechanism due to cell sensitivity to shear forces [15]. The flasks were provided pre-sterilized by the supplier. All media batching, inoculation, sampling, and culture exchange operations were

executed using aseptic technique on an open lab bench. We experienced no known contaminations over the course of multiple experiments.

The 3-day batch processes included one day of outgrowth in BMGY media, followed by two days of induction in BMMY. The growth trends for each protein are shown below. Samples taken directly from the flask were diluted by a factor of 10 to allow for accurate readings from the spectrophotometer. The G-CSF culture showed similar growth compared with the clone selection data, with the majority of growth occurring on day 1 (average = 21.4 OD_{600}). Some

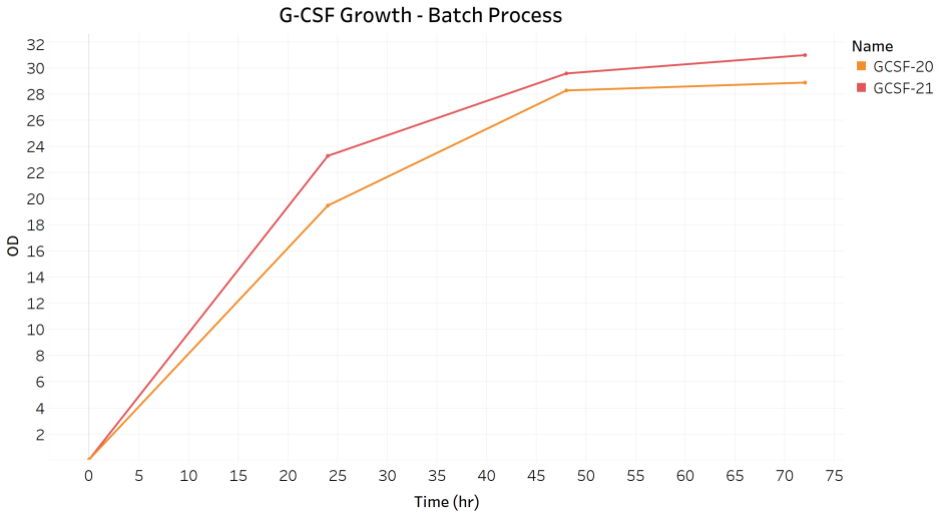

FIGURE 11. G-CSF GROWTH TREND - 3-DAY BATCH PROCESS

additional growth occurred of the first day of methanol induction (average = 7.55 OD_{600}). Minimal growth was shown in response to the methanol spike on day 3 (average = 1.00 OD_{600}). This indicates that by the second day of induction, the majority of glycerol had been depleted and cells were primarily consuming methanol as the carbon source, driving protein production over cell replication.

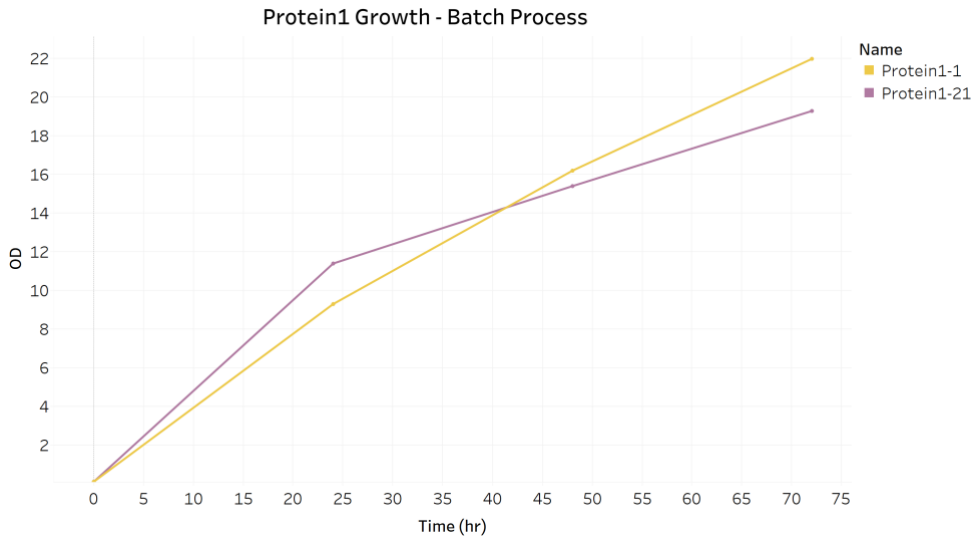

FIGURE 12. PROTEIN-1 GROWTH TREND - 3-DAY BATCH PROCESS

Protein1 showed slower growth compared with G-CSF, see trend below. This issue was traced to an error during culture inoculation. An MCB stock was used rather than the appropriate WCB for each Protein1 clone, see Appendix 2. The WCB cells undergo a 2-day shake flask operation where they are able to mature, whereas the MCB stock was immediately frozen following clone selection. The limited maturity of the cells used for this test likely resulted in the slow cell growth exhibited. For both molecules, clones showed consistent growth profiles, achieving <1.91 standard deviation for final OD measurements.

The protein titer (mg/L) was measured by SDS-PAGE analysis for each protein and clone. The gel images are shown below. Additional incremental standard concentrations were included on these gels to assist in titer estimation. Protein1 samples were run in duplicate to show repeatability. Based on the gel analysis, we estimated the protein titers shown in Table 8.

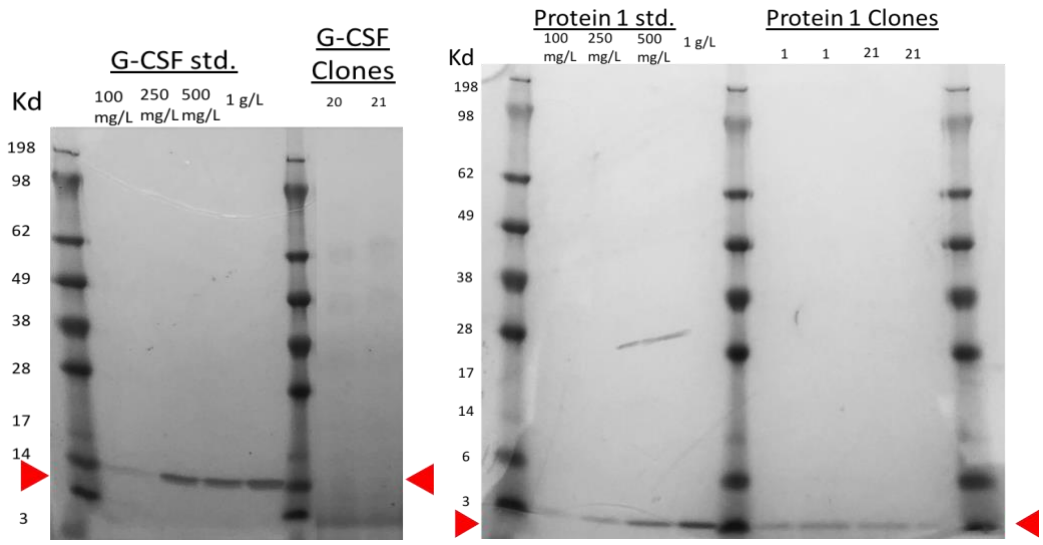

FIGURE 13. G-CSF & PROTEIN-1 SDS-PAGE GEL

Product-Clone	Estimated Titer (mg/L)
G-CSF-20	100
G-CSF-21	100
Protein1-1	300
Protein1-21	250

TABLE 8. BATCH PROCESS TITER ESTIMATES

As with the clone selection process, Protein-1 cultures achieved higher titers than G-CSF despite the use of MCB stock as inoculum. Again, this likely owes to the smaller molecular weight and overall molecular simplicity of Protein-1 compared to G-CSF. The G-CSF titer achieved is consistent with results reported by MIT Koch Institute researchers using a small-scale perfusion system [8]. A major difference from the mentioned publication, however, is that these results were achieved with Alternative Host Consortium vector materials as opposed to commercially available materials from companies such as Invitrogen. Additionally, the vectors used were provided for early stage testing and did not include much of the optimization that the Alternative Host Consortium hopes to deliver in future vector technologies. In all, achieving 100 mg/L titer for G-

CSF using an un-optimized vector and wild type *Pichia* strain should be considered a positive proof-of-concept result.

4.3. MEDIA COMPARISON TESTING

The formulation for the rich defined media (RDM) used in this experiment was taken directly from the *Biotechnology & Bioengineering* journal publication by Matthews et. al [10]. The media sciences group prepared the vitamin solution, outgrowth RDM and production RDM onsite. There were issues during the first attempted formulation with precipitation after incorporation of the PTM1 salts solution. Going forward, the salts solution was added directly to the culture shake flask immediately prior to inoculation. Agitation started immediately following inoculation, and no precipitation was observed.

G-CSF clone 20 WCBs were used for both the BMGY flask and the RDM flask. Cultures were inoculated simultaneously in the two medias. OD_{600} measurements were also taken simultaneously every 24 hours. The growth trends for the two medias are shown below. The

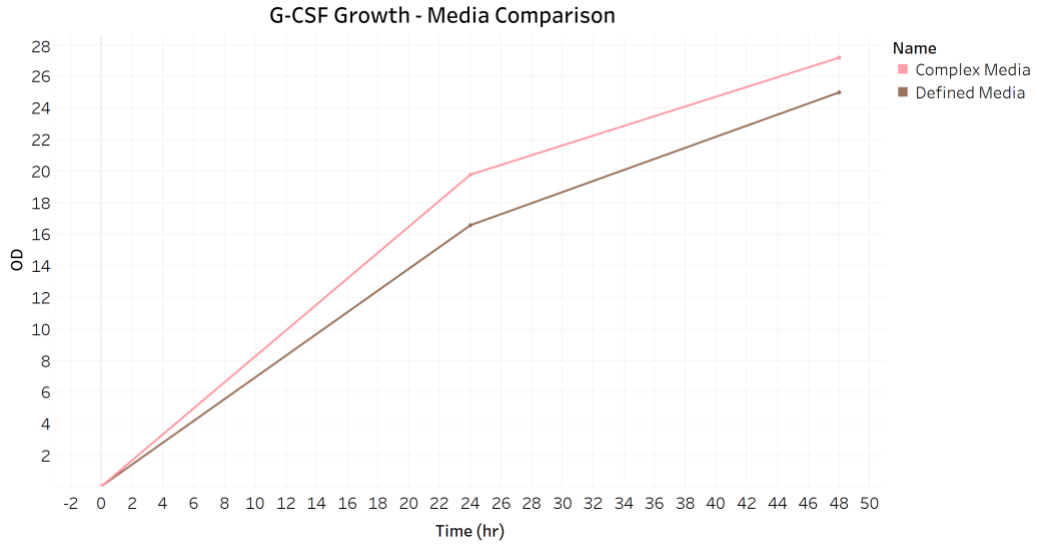

FIGURE 14. MEDIA COMPARISON GROWTH TREND

RDM culture showed slower growth during both the first 24 hours of outgrowth and methanol induction compared with BMGY. The RDM and BMGY cultures achieved final optical densities of 25.0 and 27.2 respectively, which represents a 9.2% lower final OD for the RDM culture. This result mimics results in the publication by Matthews et al, who found that RDM final OD was ~9.8% lower than BMGY (23.1 and 23.5, respectively) [10].

The cultures were harvested simultaneously after 24 hours of methanol induction. Cell broth samples were used for SDS-PAGE analysis. Expression of G-CSF was stronger in the Rich Defined Media than in the complex BMGY media. The following are the estimated titer results for the two cultures:

Media	Estimated Titer (mg/L)
RDM (Rich Defined Media)	250
BMGY (Buffered complex Glycerol Media)	100

TABLE 9. MEDIA COMPARISION TITER ESTIMATES.

The SDS-PAGE gel is shown below on the left, with the final titer samples for defined and

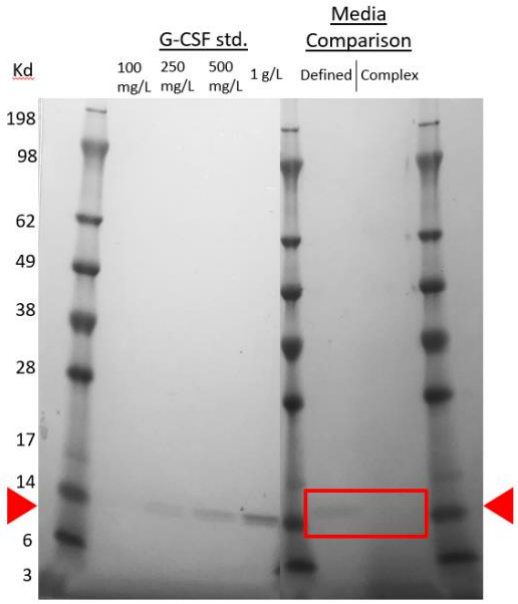

FIGURE 16. MEDIA COMPARISON SDS-PAGE GEL FINAL TITER

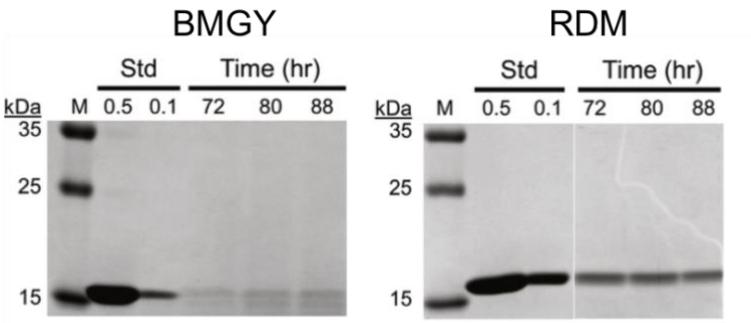

FIGURE 16. FIGURE TAKEN FROM MATTHEWS ET. AL SHOWING G-CSF TITER IN RDM & BMGY [3].

complex media highlighted by the red box. The figure on the right is taken from Matthews et. al and shows the relative titer results for RDM and BMGY cultures performed in a small scale perfusion system. Our results support the previous data, showing higher G-CSF production in defined media. Additionally, the final titer achieved in RDM was the highest of any of our batch shake flask experiments in BMGY, including those with an additional day of methanol induction.

Firstly, these results are encouraging in that they support previous published results. Secondly, they indicate that not only does defined media support *Pichia* growth, but could also potentially produce higher levels of protein expression. Increasing process productivity has driven much of the development of customized defined medias in CHO processes. Targeted media formulation development could lead to similar efficiency gains in *Pichia*. It should be noted that G-CSF is a fairly simple molecule compared with more sophisticated biologic molecules such as monoclonal antibodies. Efficiency gains shown in this RDM formulation for G-CSF may not have similar implications for other molecule types. However, the basic nutrient building blocks established with RDM offer a well-informed structure for future *Pichia* defined media development.

4.4. 250mL Fermenter Run

The Eppendorf DASBox system offered the first opportunity to run a controlled fed batch *Pichia* process over an extended culture duration (6 days). We programmed a control scheme for the experiment based on the process used in Potgeiter et. al in 2009. Using a strain engineering technology developed at a company called Glycofi, these researchers were able to produce a glycosylated monoclonal antibody molecule in *Pichia* at titers over 1 g/L [7]. Therefore, using their control scheme offered a level of confidence that we could achieve measurable protein production, and could also use their results as a comparator.

The assembled DASBox fermenter system is picture below. The two 250mL fermenters on the left and right supported cultures of G-CSF, clone 20 and Protein1, clone1 respectively. The various features of the system are summarized in the table below. Once the recipe was

IMAGE 6. IN PROCESS EPPENDORF DASBOX FERMENTER SYSTEM

System Component	Description
pH control	Black & multicolor probe, submerged
Temperature Jacket	Metal base surrounding vessels provides temp control
Addition lines	4 small clear tubing sets per vessel with top & submerged addition options
Addition pumps	Circular peristaltic pumps, back panel
Agitator	Large silver/blue drive, magnetically coupled with submerged 2-blade shaft
Overlay gas	Off-white tubing supplying air from metal base through top mounted filter
Exhaust gas	Large top mounted circular tubing/filter

TABLE 10. EPPENDORF DASBOX SYSTEM COMPONENTS

started, the system ran automatically with the exception of manual sampling operations. The pH was measured on an offline gas analyzer, and the DASBox reading was adjusted as needed. There was fairly limited pH drift over the course of the 6-day culture: (1) GCSF-20 average pH = 6.55 ± 0.07; (2) RV-1 average pH = 6.54 ± 0.08.

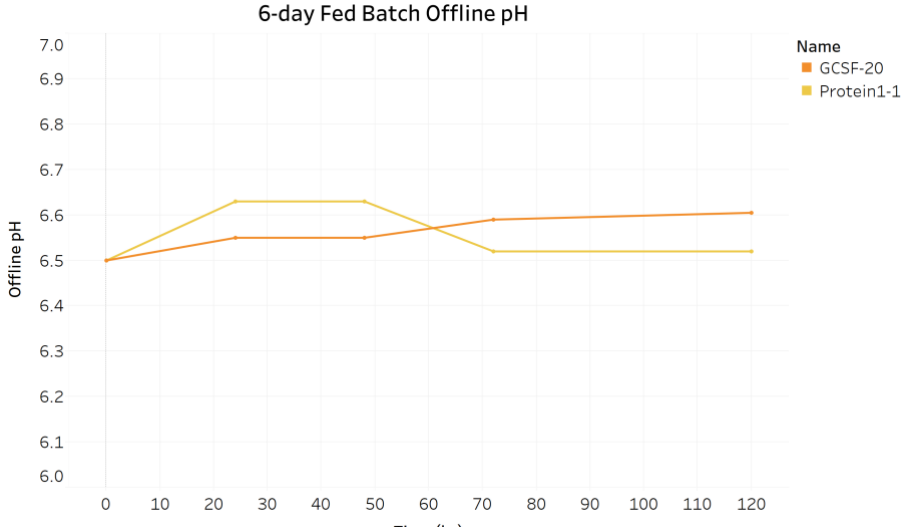

FIGURE 17. OFFLINE PH READINGS - DASBOX 6-DAY FED BATCH PROCESS

Biomass concentration was measured by wet cell weight (g/L) at various 24-hour increments throughout the process. Wet cell weight is determined by centrifuging a sample of culture and determining the ratio of cell pellet / cell broth after the separation is complete.

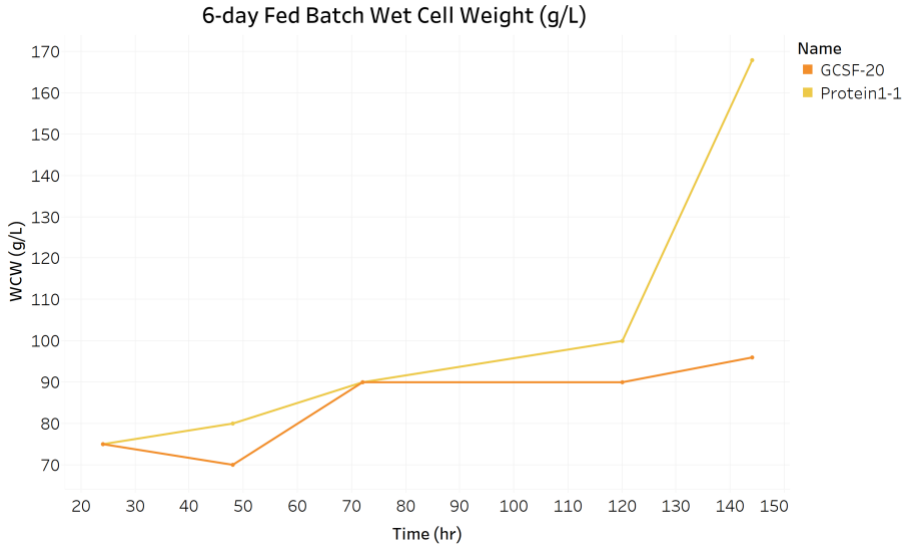

FIGURE 18. BIOMASS CONCENTRATION - 6-DAY DASBOX FED BATCH PROCESS

The biomass concentration showed a fairly steady rate of increase over the course of the culture, with the exception of the final sample for Protein-1. It is possible that this number is inflated due to sampling the culture after agitation had been terminated. If additional cell settling had occurred prior to the wet cell weight measurement, it could produce an inflated final reading.

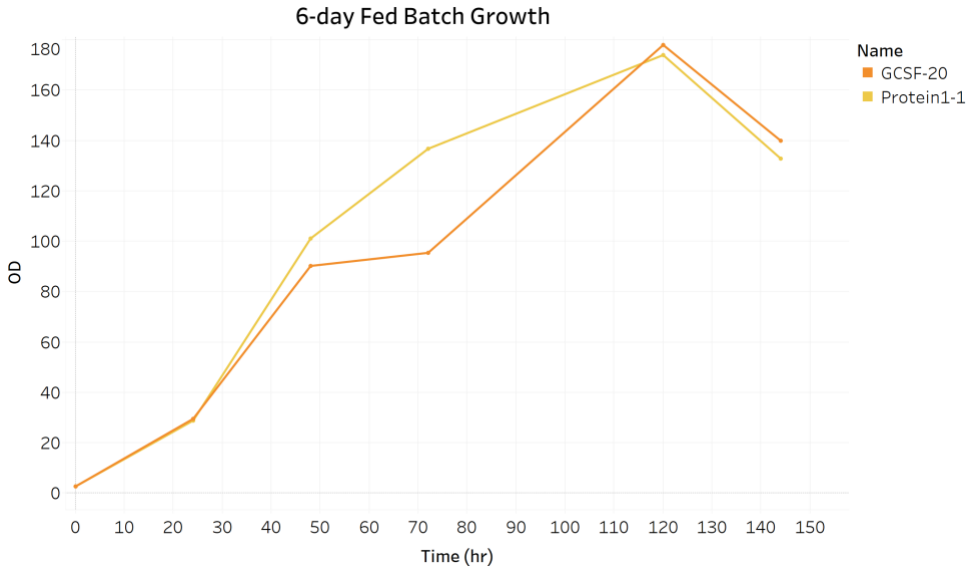

FIGURE 19. GROWTH TREND - 6-DAY FED BATCH PROCESS

OD_{600} measurements were taken every 24 hours, with the exception of day 4. Both G-CSF and Protein1 cultures reached a maximum optical density on day 5 and declined on day 6. After the vessels were disassembled following the run, there was culture-colored residue on both of the vent filters. It is possibly that foam in the vessel saturated the exhaust line after day 5, and caused a decline in overall health of the cells and therefore OD_{600}. The cultures completed with a final optical density of 140.0 and 132.9 for G-CSF and Protein-1 respectively.

The vessels were sampled and aliquots were stored at multiple time points following induction with methanol. Sample material was analyzed for titer using SDS-PAGE gel analysis.

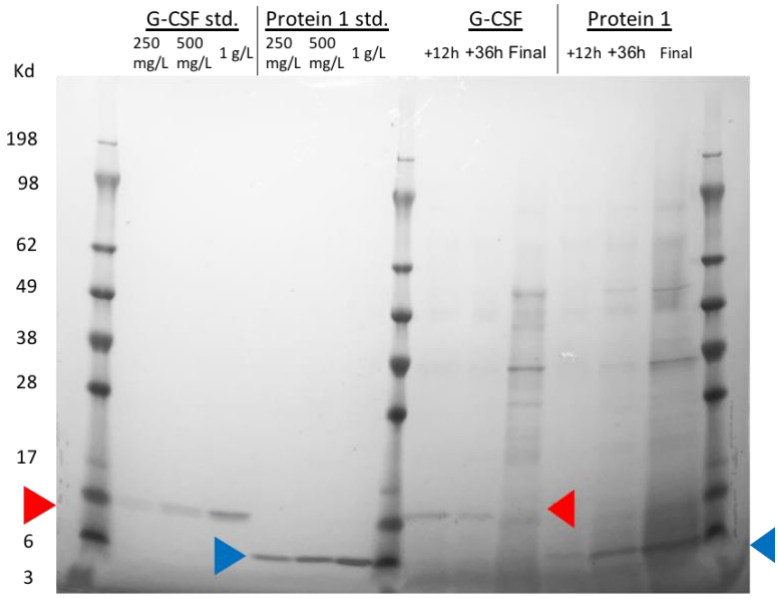

FIGURE 20. SDS-PAGE GEL, 6-DAY FED BATCH PROCESS

The time-course titer estimates are summarized in the table below, alongside the gel. Protein1 (indicated by blue arrows on the gel) showed strong titer improvement over the course of the culture, reaching a maximum of ~1g/L. However, the final titer reading for G-CSF, indicated by the red arrows, appears to decrease over the course of the run. G-CSF is prone to aggregation

Protein	Hours post induction	Estimated Titer (mg/L)
G-CSF	12	500
	36	450
	Final	300
Protein1	12	100
	36	450
	Final	1000

TABLE 11. TITER ESTIMATES, 6-DAY FED BATCH PROCESS

over extended time periods in culture, which could account for the apparent titer decline. The final sample shows a high volume of species at higher molecular weights along the gel. These species are likely endogenous proteins that are native to *Pichia*, as well as potentially aggregated G-CSF product. In past experiments, a surfactant was used to guard against this effect [8]. Limiting culture duration by harvesting earlier could also help reduce the aggregation issue, and improve both final titer and product quality. The estimated titer for Protein1 was also confirmed by another analytic method called ELISA (enzyme-linked immunosorbent assay). This method is a plate-based technique widely used for quantification of protein and antibody substances [16]. The result confirmed a final titer of 0.917 g/L, <10% different from our SDS-PAGE gel estimate.

As an initial proof of concept experiment for a fed batch process, these results constitute a success. When compared with the maximum titer values achieved in shake flask experiments, G-CSF doubled its previous performance (if measured at peak value) and Protein1 produced over triple its previous best. That productivity improvement was achieved utilizing a decades old process strategy template, with very little additional tuning. Feed strategy, oxygen sparge tuning, optimized agitation control, and inoculation ratios are just some of the levers that could be adjusted to continue to drive stronger protein production in scale-up controlled fermentation processes going forward [17]. Feed strategy in particular has been shown to have a significant impact on process performance, and should be optimized in conjunction with media development studies [15].

5. BUSINESS CASE ANALYSIS

At the core of the Alternative Host Consortium's mission is the drive to reduce the cost of developing and manufacturing complex biologic medicines. Through the use of eukaryotic microorganisms such as *Pichia*, they aim to achieve significant cost reduction throughout the entire product lifecycle. A research collaboration of scientists at Amryis, Biogen and MIT indicated that the cost per gram of monoclonal antibodies in highly efficient, concentrated fed-batch CHO processes ranges from ~ $59- $81/gram [3]. In order to provide routine treatment for patient populations such as those affected by HIV, the cost would need to drop below $10/gram [3]. This section aims to better quantify how *Pichia* could help realize those cost savings by investigating the following topics: (1) Manufacturing process differences from existing hosts; (2) Impact to Cost of Goods Manufactured (COGM); (3) Other considerations including product commercialization timelines and capital investment.

5.1. PICHIA MANUFACTURING PROCESS OUTLINE

The *Pichia* manufacturing process differs from traditional CHO manufacturing in 3 key ways, all of which have been touched on previously: (1) Faster growth in upstream fermentation steps; (2) Elimination of viral contamination risk; (3) Lower impurity levels allowing for potentially simplified downstream operations [2]. The key difference between *Pichia* and *E. coli*, another potential alternative host, is the fact that *Pichia* secretes the product extracellularly, whereas *E. coli* processes require arduous downstream steps to extract the product from inside the cell wall [4]. The differences are outlined in the process flow map below, which depicts *Pichia* as a hybrid of the *E. coli* and CHO manufacturing processes. It combines the speed of *E. coli* upstream

operations with a simplified version of CHO downstream operations, owing to extracellular secretion of the product.

The upstream durations depicted are averages from the noted sources and may vary widely depending on specific product requirements. However, *Pichia's* ability to double ~10 times faster than CHO ultimately allows for faster accumulation of biomass that limits the speed of CHO cell

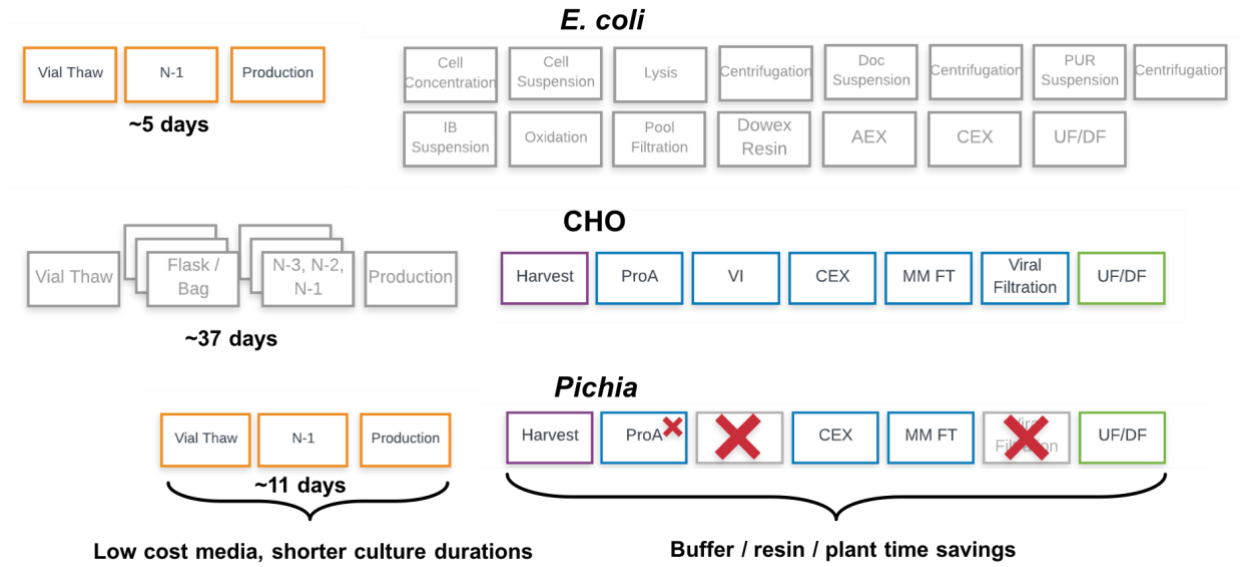

FIGURE 21. GENERALIZED BIOMANUFACTURING PROCESS OUTLINES BY UNIT OPERATION; E. COLI, CHO, PICHIA [3], [7], [8]

culture operations [2]. As previously discussed, *Pichia* provides the opportunity to eliminate viral inactivation and filtration steps typical in CHO-based processes [11]. While there have been prior regulatory successes in removing these steps, the viral contamination concerns must be assessed on a per product basis. Other factors outside of the host could warrant inclusion of viral reduction steps. Examples could include inadequate facility environmental controls, raw material screening or multi-product facility complexities. The final major difference is the potential elimination of a downstream affinity chromatography step, depicted here as a small red "X" on the ProteinA unit operation. Due to the limited size of the *Pichia* secretome, relatively low levels of host cell protein (HCP) and other related impurities have been observed following fermentation. As the understanding of specific *Pichia* HCP expression grows, alongside advanced strain engineering

techniques, the ability to further refine and reduce the impurity load entering the downstream process has the potential to improve even further than the current >80% purity observed in some perfusion processes [2]. The current product quality performance level warrants consideration that a simplified 2-stage chromatography downstream process may be suitable for future *Pichia* processes, however, this remains to be demonstrated. For these modeling purposes, the best case scenario of a simplified downstream process was used.

5.2. COST OF GOODS MANUFACTURED (COGM) ANALYSIS

Using the unit operation process outlines described above, our next goal was to quantify the potential cost advantage of a simplified *Pichia* process to produce mAbs on the basis of cost of goods manufactured, or COGM. We present a simplified formula for estimating COGM that accounts for raw material, consumable, labor and facility costs associated with producing a hypothetical product. In the absence of publicly available cost analyses for a mature *Pichia* biologic process, we developed an analysis based on an existing well-understood model for a CHO monoclonal antibody (mAb) process. By accounting for the expected efficiency gains in process simplification and operational speed, we provide an estimate for the relative potential savings of a *Pichia*-based process with respect to a generalized CHO baseline.

5.2.1. PICHIA COGM PROJECTIONS

Detailed cost information for commercially available biologic products are not readily disclosed by biotech and pharmaceutical companies. There are privacy concerns around raw material costs/quantities, supplier agreements and process-related intellectual property that warrant protection of this type of information. A detailed breakdown of costs by unit operation (e.g. upstream, purification, formulation) would further increase these concerns. In the absence of this type of specific data, here we developed a generalized example of a typical CHO process based

on cost modeling sources for the biotechnology industry as a whole. The following are the key assumptions we used:

Facility	
Format	Small-scale single use facility
# of Vessels	6 x 2,000L
Lots per year	6 x 22 per vessel = 132 / year Average 13-day production cycle w/ short maintenance shutdown[18]
Annual Costs	
Facility expenses (Operating, Depreciation, etc.)	$12.5M / year [18]
Labor	$20M / year (250 staff) [18]
Raw Materials / Consumables	$22M / year Cost ratio for single-use format; (60% Facility & Labor / 40% Raw material) [18], [19]

TABLE 12. CHO COST MODEL FOR SINGLE-USE 6 X 2,000L FACILITY

We selected a small-scale single-use facility model to align with a growing trend towards agility and flexibility in new biotechnology manufacturing facilities. Single-use technologies have proven to be cost effective by reducing initial capital investments and shrinking facility footprints [20]. This cost-focused strategy aligns with the overall goal of the Alternative Host Consortium to drive significant cost reduction throughout the entire biologics product lifecycle.

Our aim was to then adapt this case study CHO model to an estimated *Pichia*-based model. That required segmentation of the per lot raw material costs into the various unit operations. We utilized relative cost percentages outlined in Mitchell-Logean *et al.* to estimate the relative unit operation costs [19]. Then, based on the manufacturing process flow described in section 5.1, we estimated the expected costs for the same process on a *Pichia* host platform. The results of a per

lot raw material cost comparison between a CHO and *Pichia* platform for a mAb product are summarized below. The overall mass produced per lot is not defined here, but is incorporated in the analysis in the following section.

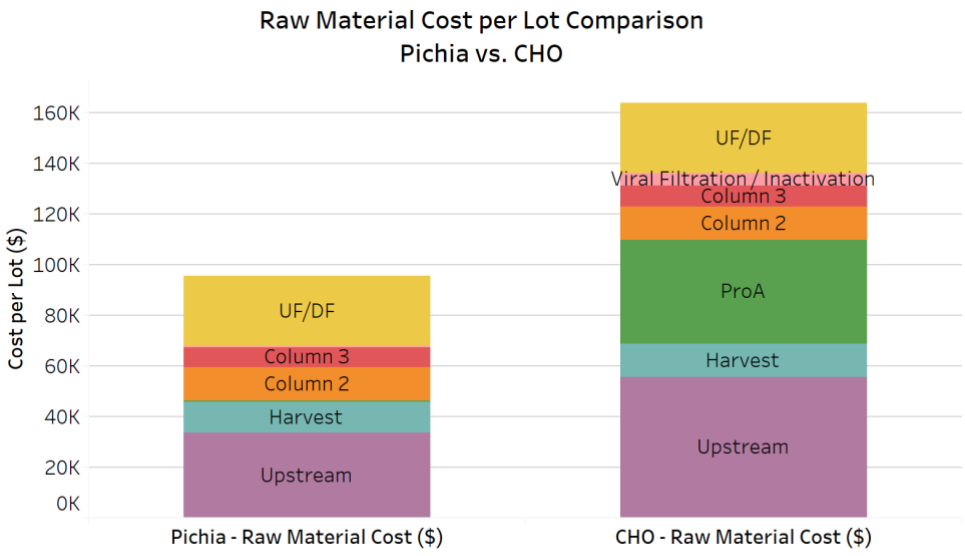

FIGURE 22. RAW MATERIAL COST PER LOT COMPARISON

The key adjustments made for the *Pichia* case were the removal of Protein A chromatography, removal of viral filtration and inactivation steps, and the conservative estimate for upstream cost reductions to 60% of CHO costs based on shorter culture durations and higher cell densities. In all, these differences amounted to ~42% reduction in raw material costs per lot from ~ $164.1K to ~ $95.9K. While this assessment is limited by the assumptions we made for the base CHO model, the potential range of savings and unit operation contributions can be used to shape expectations for future *Pichia* product cost models.

5.2.2. PRODUCTIVITY AND DEMAND ANALYSIS

While raw material costs are certainly a crucial aspect of overall costs, they can easily be eclipsed by operational cost savings depending on the scale and productivity of a given process. Going one step further than the per lot raw material savings, here we attempt to quantify the

potential savings afforded by the **speed** of *Pichia* processes. Based on the assumptions outlined in the previous section, we utilize the following formula to develop a Total Cost per Lot value that includes both raw materials/consumables and operational costs.

$$Total\ Cost\ per\ Lot\ =\ \boldsymbol{Raw\ Material}\frac{\$}{\boldsymbol{Lot}} + (Fixed\ Operational\frac{\$}{day} * \boldsymbol{Run\ rate\ (days)})$$

EQUATION 1. TOTAL COST PER LOT

The values defined in our CHO model provide the baseline costs outlined below. For our *Pichia* case, we fixed the operational cost per day as a conservative assumption that a *Pichia* facility would require similar labor and operational overhead expenses to CHO. The "run rate" values represent the cadence at which a given facility completes a single lot. For CHO, using the 13-day production cycle as the limiting factor, annual production of 132 lots equates to a 2.77 day "run rate". *Pichia*, due to the faster cell growth in upstream, can achieve a much shorter production cycle of ~ 7 days [7]. The production fermenter cycle time, however, is not always the limiting bottleneck to the speed of the end-to-end process. There could be downstream steps, equipment turn-around activities, or limiting media/buffer supply systems that also limit facility run rates. So, for the purposes of this model, we included three hypothetical run rate scenarios for our *Pichia* process: (1) *Pichia* matches existing CHO run rate of 2.77 days; (2) *Pichia* achieves 1.5X CHO run rate; (3) *Pichia* achieves 2X CHO run rate.

	CHO	**Pichia**
Operational cost / Day ($/Day)	$89.04K/day	
Raw material Cost / Lot ($/Lot)	$164.1K/lot	$95.9K/lot
Run Rate (days)	2.77d	Worst Case = 2.77d
		1.5X CHO = 1.84d
		2X CHO = 1.38d

TABLE 13. OPERATIONAL COST ESTIMATES PICHIA VS. CHO

Using this framework, we then wanted to see whether such a *Pichia* process would be cost competitive with CHO when applied to a hypothetical product demand scenario. Two additional variables needed to be assigned for this exercise: process productivity (product mass per run), and annual demand in product volume. To start, we utilized the following average values [18]:

1. Titer = 5g/L
2. Downstream Process Yield = 70%
3. Annual Demand = 0.1 metric ton (or 100 kilograms)

Initially, we assumed the *Pichia* process would be able to match CHO in process productivity, i.e. achieving equal titer and process yields (we will model lower *Pichia* productivity in upcoming examples). The resulting cost analysis is summarized below for the CHO baseline and the 3 different *Pichia* run rate scenarios.

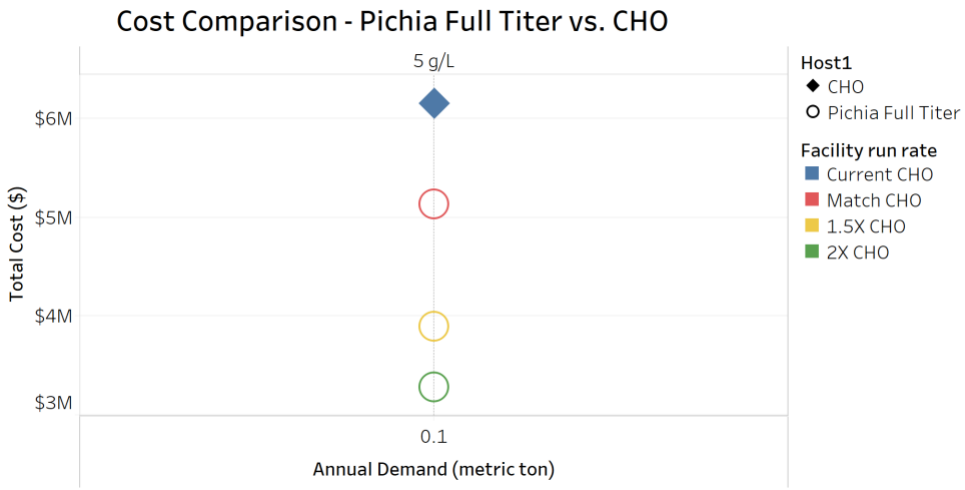

FIGURE 23. INITIAL COST COMPARISON ANALYSIS PICHIA VS. CHO

The total cost for CHO is represented by the blue diamond. For each of the *Pichia* run rate scenarios, the total annual cost is represented by the colored circles. For the "Match CHO" run rate scenario, the annual cost savings compared to CHO total ~ $1.02M. That difference solely represents the lower raw material costs, as the run rate remained equal to CHO. However, as you

begin to model faster run rates (1.5X and 2X), you see the additional savings significantly increase, resulting in ~$2.87M in annual savings for the 2X run rate scenario.

A major caveat as we continue to work with this model, is that we are only assigning operational plant costs to the days it would take to produce the given product demand volume. Any additional days in the year would also carry operational cost, but we assume that other products or budget would be accounting for those costs. Put simply, by assuming less plant time for faster run rate *Pichia* processes, we are assuming there would be other product demands to fill the newly available plant days. The validity of that assumption depends heavily on the capacity utilization, product mix, and product/site licensing realities of the manufacturing network of a given company. Application of this model to a commercial scenario should consider these factors as appropriate.

In addition to the demand scenario modeled above, we wanted to perform a sensitivity analysis on both the process productivity and the annual demand. This analysis serves to identify the type of product that could generate the most cost benefit by considering a *Pichia* host platform. To perform this analysis, we considered the following values to try to encompass the range of current marketed biologics [18]: (1) Titers (g/L), [2, 5, 10, 20]; (2) Annual Demand (metric ton), [0.02, 0.1, 2, 5]. The resulting cost landscape is summarized in the chart below.

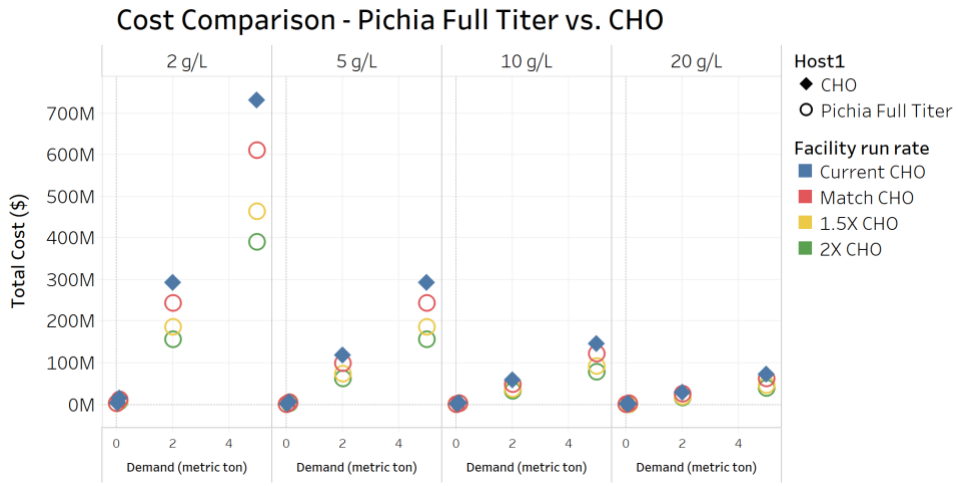

FIGURE 24. TITER AND DEMAND COST SENSITIVITY ANALYSIS

As you move from left to right across the chart, the relative productivity of the process increases, reducing the number of lots required to meet each annual demand range and reducing total cost. For example, producing 100 kilos of product requires 36 lots at 2 g/L, but only 4 lots at 20 g/L. With fewer CHO lots required to meet demand, the savings effect of a faster run rate is diminished and the absolute value of savings provided by *Pichia* become smaller. Increases in demand, however, show *Pichia* savings scaling along with increasing operating costs. Therefore, a low productivity / high volume product could prove an attractive area for consideration of *Pichia*.

The low productivity bracket is also likely the most realistic in terms of *Pichia*'s current performance level. Around 1 g/L represents the high level of the titer range for monoclonal antibody production in *Pichia*, whereas current clinical stage CHO-based mAb products are achieving 10-20 g/L depending on process format [6][3]. Given that significant performance improvement is likely required to match current CHO titers, we investigated whether the cost savings described above could be achieved if *Pichia* continues to produce at lower titers. Specifically, we repeated the prior analysis with the condition that *Pichia* only achieved **1 g/L** per previously published results [6]. The results are summarized below for two annual demand

scenarios (0.02, and 0.1 metric tons/year). The three *Pichia* run rate scenarios are now represented by the green, yellow, and red bars. Various possible CHO titers are shown as blue bars on each graph to help illustrate the cost advantage of high titer CHO processes.

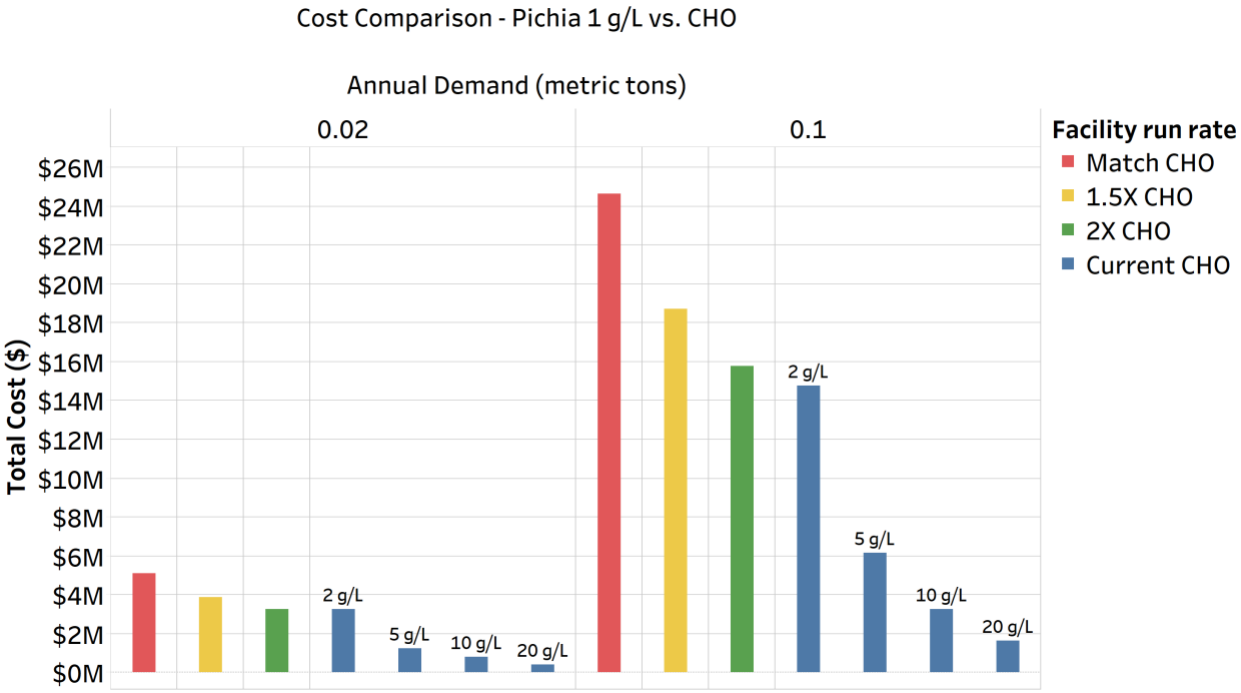

FIGURE 25. COST COMPARISON OF PICHIA 1 G/L PROCESS VS. CHO AT VARIOUS TITER/DEMAND RANGES

Here, at 1g/L titer, *Pichia* does not provide a cost benefit compared with any of the CHO titer scenarios. Further modeling showed that at the 2X run rate, *Pichia* must perform at ~50% of CHO titers to achieve equivalent costs. The productivity performance in *Pichia* should therefore continue to be prioritized as it is critical to achieving cost reduction goals.

Lastly, we wanted to include a sensitivity analysis around the exclusion of the Protein A affinity chromatography step. As the current data is limited surrounding the potential removal of this downstream step, we performed the analysis with the assumption that *Pichia* would require

Protein A chromatogray. The raw material cost per lot would increase from $95.9K to $136.9K, resulting in a 17% reduction from CHO. The relative operational savings are therefore slightly

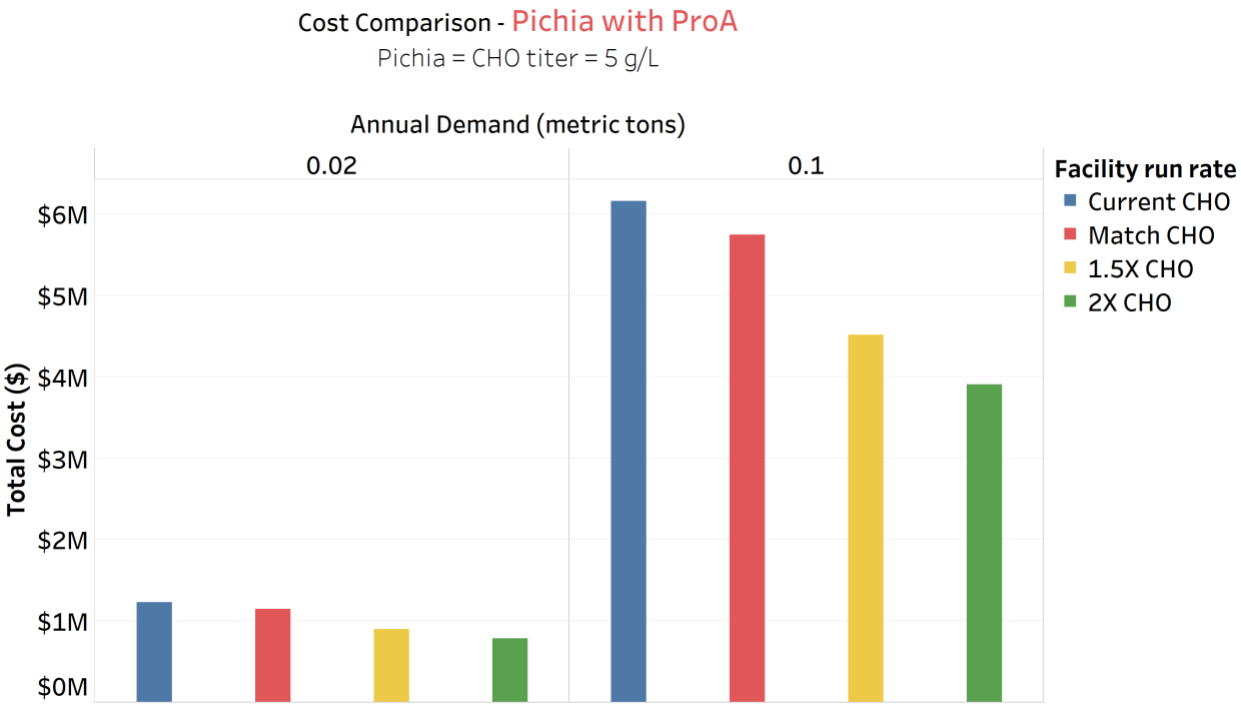

FIGURE 26. PICHIA WITH PROTEIN A - COST ANALYSIS

diminished. The chart below outlines the total cost savings for the scenario presented earlier. Ultimately, including Protein A in the *Pichia* process will result in an additional $41K/lot raw material expense. Despite reduced raw material savings, *Pichia* still provides a total cost benefit especially at faster cycle times.

6. NEXT STEPS & RECOMMENDATIONS

This project accomplishes two initial steps in the establishment of a *Pichia* host production system at Amgen:

1. It provides proof-of-concept data with simple target molecules to benchmark the baseline performance capability of the *Pichia* host system.
2. It outlines a framework for understanding the potential cost advantages compared with CHO, highlighting the importance of simplified downstream processes and reduced time for operations in a facility as important drivers of potential savings. To best realize these advantages though, gains in titer will be needed as well.

While these steps are critical in qualifying the current state and potential of this technology, there are additional questions that must also be addressed before *Pichia* could be adopted by large biopharmaceutical companies currently operating highly optimized CHO-based manufacturing networks.

First, *Pichia* needs to be tested with more complex molecules that offer challenges such as higher molecular weight (mAb or larger) or that require multiple glycosylation sites. The results achieved with G-CSF and Protein1 are encouraging in that they match existing published titer results, and show that 2-3x titer improvement can be acheived with implementation of simple process control strategies. However, these molecules are relatively small and structurally simple in comparison to the rapidly advancing complexity of future biologics drug candidates.. A benchmarking of current *Pichia* performance level is therefore needed with a range of complex molecular structures.

The results discussed here also only represent half of the product process development strategy. We executed these studies through the upstream and harvest unit operations. What

remains is the task of downstream purification and formulation process development. It is in these studies where the argument for removal of Protein A purification can be quantifiably tested. By documenting the purity profile of a given *Pichia* process, the downstream unit operations can be defined. An accurate estimate for overall downstream yield based on the number and type of chromatography steps required could further inform the business case analysis performed here, if significantly different from current CHO performance.

Our Eppendorf fermenter test took a step towards establishing a controlled scalable manufacturing *Pichia* process at Amgen. Additionally, our Rich Defined Media testing established a baseline media formulation for future scaled production. However, both the media and process control strategy may benefit from further optimization through DOE testing to establish best practices for *Pichia* processes moving forward. A major shift in terms of media testing will be the potential replacement of methanol as the induction carbon source. Due to safety concerns, alternative carbon sources could avoid potentially expensive safety controls for methanol containing cultures.

Productivity improvement will be a key metric to drive process and media optimization studies. As shown by the cost analysis, *Pichia* will need to show meaningful improvements on current titers for MAbs to be financially competitive with CHO-based processes above 2 g/L. These gains are achievable through many levers. Prior industry efforts on *Pichia* process intensification have resulted in ~2x productivity improvement [2]. The rich defined media achieved ~10x titer gains across multiple product classes in prior experiments [10]. In addition to these in-process efforts, strain engineering has the promise to provide an additional productivity boost. The Alternative Host Consortium is utilizing a deep genetic understanding of the *Pichia* secretion process to develop highly efficient product-secreting strains as source material for their

industry partners. With focused efforts on each of these fronts, *Pichia* titers could improve much the same way that CHO titers improved over years of industry process optimization as illustrated in the graph below [21].

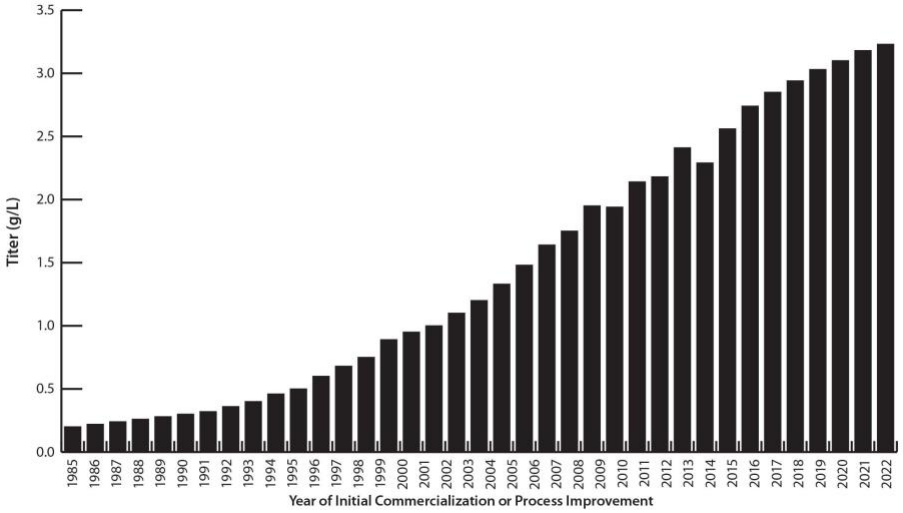

FIGURE 27. AVERAGE COMMERCIAL SCALE MAB TITERS, 1985-2023. FIGURE 1, SOURCE [18]

Lastly, the cost analysis presented will need to be re-assessed with respect to specific manufacturing facility capabilities and cost structures. The model presented is highly dependent on the potential achievable run rate at the commercial production scale. To arrive at an appropriate run rate estimate, individual facilities must be modeled based on their upstream/downstream capacity and relative unit operation turnover cadences. Additionally, the realistic capital investment requirements must be assessed based on manufacturing network capabilities of owned and outsourced capacity. An existing microbial facility will require a significantly different up-front investment compared with a retro-fit of a CHO-based facility. An option analysis must be performed to quantify the potential capital need of available facilities, weighted by their future run rate capability and cost profile.

Another limitation of this cost model is that it focuses on a standard fed-batch process. Other intensified process formats such as perfusion or continuous manufacturing have the potential

to increase capacity utilization efficiency even further by eliminating equipment downtime. Researchers at MIT have already developed a 3L continuous manufacturing platform for protein expression in *Pichia*, and have successfully produced multiple therapeutic molecules [8]. Development and scale-up of this technology for commercial use would require additional assessment to account for cost and run-rate advantages.

Overall, *Pichia* offers an entirely new operational challenge for the scientists, engineers and strategists at Amgen to consider. By establishing an early understanding of the process challenges and cost reduction promise of the *Pichia* host, the Cell Line Development team can better understand the strategic advantage of utilizing *Pichia* going forward. The key takeaways from the analysis presented here are the following:

1. *Pichia* may provide raw material/consumable cost savings compared with equivalent titer fed-batch CHO processes through process simplification (e.g. elimination of viral inactivation, Pro A, or some upstream process steps).
2. *Pichia* based processes may be cost competitive at titers ~50% lower than equivalent CHO-based processes due to the potential for improved facility utilization at faster run rates.
3. Alternative facility configuration or integrated process strategies may also provide cost benefits, but were not specifically examined in this study.

By understanding these cost drivers, the key components of future *Pichia* processes can be challenged early in the product development lifecycle to support informed decisions around host selection.

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APPENDIX

A.1 MEDIA COMPONENT BREAKDOWN

Yeast Extract – Peptone – Dextrose (YPD Media)

Compound	Quantity per L	Unit
Yeast extract	10	g
Peptone	20	g
Dextrose	20	g

BMGY – Buffered Glycerol Complex Medium

Supplier: Teknova
Item Number: B8000

Compound	Concentration
Yeast extract	1%
Tryptone	2%
Yeast Nitrogen Base	1.34%
Biotin	0.4 mg/mL
Glycerol	1%
Salts Buffer	10%

BMMY- Buffered Methanol Complex Medium

Supplier: Teknova
Item Number: B8100

Compound	Concentration
Yeast extract	1%
Tryptone	2%
Yeast Nitrogen Base	1.34%
Biotin	0.4 mg/mL
Methanol	1.5%
Salts Buffer	10%

Custom Rich Defined Media (RDM)

This media formulation is based on a previously published *Pichia* media optimization study [10]. The vitamins solution was prepared separately and utilized as needed in Outgrowth and Production RDM formulations.

Vitamins solution

Compound	**Quantity per L**	**Unit**
Biotin	0.05	mg
Calcium Pantothenate	1	mg
Nicotinic Acid	1	mg
Inositol	25	mg
Thiamine HCl	1	mg
Pyridoxine HCl	1	mg
Para-aminobenzoic acid	0.2	mg

Pichia RDM ***Outgrowth*** Media

a. Prepare appropriate volume of media per the following formula:

Compound	**Quantity per L**	**Unit**
Glycerol	40	mL
KH2PO4	12.00	g
MgSO4.7H2O	4.70	g
CaCl2.2H2O	0.36	g
(NH4)2SO4	1.65	g
KOH	3.37	g
Glutamine	1.74	g
Arginine	1.46	g
Vitamins	3.33	mL
Lipids	10.00	mL

b. Immediately prior to inoculation, add 4.35 ml/L of PTM1 Salts solution.

Pichia Custom Defined ***Production*** Media

a. Prepare appropriate volume of media per the following formula:

Compound	Quantity per L	Unit
KH2PO4	12.00	g
MgSO4.7H2O	4.70	g
CaCl2.2H2O	0.36	g
(NH4)2SO4	1.65	g
KOH	3.37	g
Glutamine	1.74	g
Arginine	1.46	g
Vitamins	3.33	mL
Lipids	10.00	mL

b. Immediately prior to inoculation, add the following:
    i. 4.35 mL/L PTM1 Salts Solution
    ii. 1.5% v/v Methanol